Melanophore-concentrating and melanophore-dispensing peptides of teleost pituitary.

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MELANOPHORE-CONCENTRATING AND MELANOPHORE-DISPERSING PEPTIDES OF TELEOST PITUITARY

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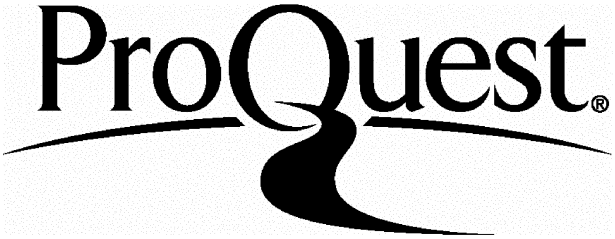


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ABSTRACT

The physicochemical properties of the melanophore-concentrating hormone (MCH) of teleost pituitaries were studied. It was found to be a heat-stable trypsin-sensitive peptide of about 1 700 daltons which was similar in charge, size and hydrophobicity to α-MSH. Partial purification by cation-exchange, gel-filtration and HPLC showed that it was not identical with any of the melanocyte-stimulating hormones (MSH's) of teleost pituitary, but the possibility that these peptides are structurally related could not be excluded. Both hypothalamic and pituitary forms of MCH were distinguished from the known neurohypophysial hormones by their size and their insensitivity to reduction with thiol-reagents. Complete purification of α-MSH's showed that these were present in greater amounts than was MCH in the pituitary. The implications of this are discussed recommending the hypothalamus rather than the pituitary for the purification of MCH. Since melanophore-concentrating activity could be masked in crude hypothalamic extracts, MCH may have a wider phylogeny than has been previously appreciated.

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List of Abbreviations

ACTH	adrenocorticotrophic hormone
AVT	arginine vasotocin
B	black background
BSA	bovine serum albumin
CA	C-terminal amino sequence ion
CAlk	C-terminal alkyl sequence ion
CCK	cholecystokinin
ConA	concanavalin A
DnsCl	N,N-dimethylaminonapthalene-5-sulphonyl chloride
EGTA	ethyleneglycol bis(β-aminoethyl)N,N'-tetraacetic acid
FAB	fast-atom bombardment
HPLC	high-performance liquid chromatography
IT	isotocin or icthyotocin
IU	international unit
MCH	melanophore-concentrating hormone
MI	melanophore index
MSH	melanocyte-stimulating hormone or melanotropin
m/z	mass/charge
NA	N-terminal amide sequence ion
NH	neurohypophysis
NIL	neurointermediate lobe
ODS	octadecyl trichlorosilane
PAS+	periodic acid-Schiff positive
PbH+	lead-haematoxylin positive
PD	pars distalis
PI	pars intermedia
TFA	trifluoroacetic acid
TLC	thin-layer chromatography
TPCK	L-1-tosylamide-2-phenylethyl chloromethyl ketone
TU	trout unit
Ve	elution volume
Vo	void volume
W	white background

CHAPTER 1

INTRODUCTION

The spectacular ability of certain animals to change their colour has intrigued man for centuries. The process is brought about by the redistribution of pigment organelles within specialised integumental cells of neural-crest origin known as chromatophores. In lower vertebrates colour-change is largely an adaptive measure to maintain camouflage despite changes in background. The predominant pigment is melanin, and animals appear pale when pigment is concentrated centripetally around the nucleus of the melanophore, and dark when it is dispersed centrifugally throughout the cytoplasm.

Colour-change by alteration of the intracellular distribution of pigment is known as physiological colour-change as distinct from morphological colour-change where changes in the number and pigment content of melanophores also occur. This report will be primarily concerned with physiological colour-change and any unqualified reference to "colour-change" will imply the physiological type.

1.1 The role of the pars intermedia in background adaptation

The pars intermedia (PI) of the amphibian pituitary has long been known to be the source of melanophore-

dispersing hormones, and injection of pituitary-extracts causes melanophore-dispersion. Teleosts however, may either pale or darken when injected with pituitary extracts depending on the species of donor and recipient (see Pickford and Atz 1957). They are also unique in having two histologically distinct cell-types in the PI; these are named according to their reaction with lead-haematoxylin (PbH) and periodic-acid-Schiff (PAS) stains as the PbH+ and PAS+ cells (Stahl, 1958). (Salmonids do not have a PAS+ cell but a chromophobic cell-type which may be an homologue of the PAS+ cell (Ball and Baker 1969)). Stahl (1958), to explain the dual character of teleost pituitary extracts, postulated that these cell-types were the sources of antagonistic melanophore-controlling hormones. Since then, the cytophysiology of the PI has been studied in relation to background (Tab 1). The PI cells show evidence of increased secretory activity during adaptation to B, and decreased activity during adaptation to W; the cell-type affected depends on the species. However, in no case was PI cell activation associated with W adaptation and therefore Stahl's hypothesis is no longer tenable.

a) The+PbH+ cell

The increased granulation seen in the PbH+ cell in B-adapted Anguilla soon after transfer to W was shown to be correlated with an increase in bioactive MSH-

Table 1. The response of the pars intermedia cells of teleosts to black background.

Group 1 PbH+ cell activated predominantly	Anguilla, Salmo, Phoxinus, Tinca
Group 2 PAS+ cell activated	Poecilia, Cichlasoma, Blennius
Group 3 PbH+ and PAS+ cell activated	Sarotherodon[1], Boops[2], Scorpaena[3]
Group 4 Neither cell type activated	Ictalurus, Heterpneustes

The PI cells become activated only in response to black background, transfer to white background causes regression.

Salmo could belong to group 3 as the response of its "PAS+ cell" (actually a chromophobe -see text) has not been determined. The group classification is that of Ball and Batten (1981) except that I have included Ictalurus and Heteropneustes as an extra group. The information for groups 1, 2 and 4 is from Ball and Baker (1969), Baker and Ball (1970) and Baker (1981). It may be significant that in Anguilla, Ictalurus and Heteropneustes, where innervation of melanophores is rudimentary, they respond to teleost pituitary extracts by dispersion and not by concentration as do most of (if not all) the others in this table.

1. Van Eys(1980), 2. Malo-Michele (1977a), 3. Malo-Michele (1977b).

content of the neurointermediate lobe (NIL)- indicating that this cell-type is the melanotrope (Baker, 1972). In this species, melanophore dispersion is associated with PbH+ cell activation as seen when this is provoked or suppressed independently of background by injections of drugs such as metopirone or reserpine (which cause activation) and 6-hydroxy-dopamine or cortisol (which cause regression)(see Holmes and Ball, 1974).

The reactivity of the PI cells towards antisera raised against mammalian α-MSH and ACTH is confined to the PbH+ cell confirming its identity as the melanotrope (Follenius and Dubois, 1974, 1980; Romain et al, 1974; Malo-Michele et al, 1976; Olivereau et al 1976). This is even true of species such as Poecilia where the PAS+ cell is active on B (Dr. B. Baker, personal communication).

b) The PAS+ cell

Although the PAS+ cell is clearly activated by B in teleosts such as Poecilia and Sarotherodon, the nature of its secretory product(s) is unknown.

In Sarotherodon the PAS+ cell reacts with mammalian prolactin antisera, but this immunoreactivity is not attributable to teleost prolactin (Rawdon 1979). In

hypophysectomised Fundulus, chronic administration of sheep prolactin causes melanization of dorsal melanophores (Pickford and Kosto, 1957). Although there might not be a causal link between these observations, they constitute circumstantial evidence for the secretion of a prolactin-like melanotropic hormone (not prolactin) by the PAS+ cell, which is active in morphological colour-change.

In species where both PI cell-types are active on B, their secretory products may interact in some way or complement each-others' action. In this regard it is noteworthy that Pickford and Kosto (1957) also found that β-MSH caused recruitment of melanophores (i.e. was complementary to prolactin).

Notably, Sarotherodon prolactin is 24 residues shorter than its mammalian counterpart (Farmer et al, 1977), and the PAS+ cell's immunoreactivity may mean that it contains a variant prolactin which is more akin to the mammalian hormone.

1.2 Physiological colour-change in teleosts

a) The role of nerves

Physiological colour-change in many teleosts is very rapid, adaptation to background reversal often being accomplished in a few minutes. This is because their

melanophores (unlike those of elasmobranchs and amphibians) are controlled predominantly by nerves. Many investigators have demonstrated the potent concentrating effects of noradrenaline, dopamine and adrenaline - both *in vivo* and *in vitro* - and there is little doubt that concentration is evoked by the release of a catecholamine neurotransmitter from sympathetic nerve-terminals close to the melanophores, which acts on α-adrenoceptors (see Bagnara and Hadley 1973).

There is also evidence that in some species, dispersion is assisted by nerves which may act via β-adrenoceptors. Thus, injections of β-adrenergic agonists caused dispersion in the freshwater angelfish *Pterophylum eimekei* (Finnin and Reed, 1970), and low doses of natural catecholamines (especially adrenaline) as well as synthetic β-agonists, caused dispersion in *Poecilia reticulata* (formerly *Lebistes reticulatus*) (Miyashita and Fujii, 1975). The presence of β-receptors which can mediate dispersion does not necessarily indicate the existence of dispersing innervation; neither does the dispersing effect of certain types of electrical-stimulation, as pointed out by Fujii and Novales (1969a). No support has emerged for the early notion of Parker (1948)that acetylcholine was the dispersing neurotransmitter.

b) The role of hormones

Melatonin is well known for its paling action on larval amphibians. It is also active in certain teleosts. The melanophores of embryonic and larval forms of Fundulus concentrate in response to melatonin both in vivo and in vitro, although adult forms are insensitive (Nichols et al, 1966; Fain and Hadley, 1966). Even the adult forms of some species (e.g. Salmo gairdneri) (Hafeez, 1970) respond to melatonin, but only in vivo. The absence of an in vitro response (Reed et al, 1969) indicates that the action is indirect - perhaps mediated by nerves; or, considering the very high doses necessary for the in vivo response - it could be a stress reaction. Although variations in plasma melatonin concentration have been observed in response to illumination (Owens et al, 1978, in Salmo gairdneri), such variations do not occur in response to background.

These responses to melatonin may be the vestige of an archaic mechanism of colour-change where melatonin was once a paling hormone, but it is unlikely that melatonin has a direct hormonal role in todays teleosts.

Although fish may exhibit pallor in response to intense stress - presumably due to adrenaline and noradrenaline from the chromaffin cells, it is considered

unlikely that this mechanism is involved in adaptative physiological colour-change.

Pituitary hormones on the other hand, do appear to be involved in physiological colour-change. The innervation of teleost melanophores and their extreme sensitivity to catecholamines has hindered the elucidation of the role pituitary hormones play in their control; this - and their great diversity as a phylogenetic group - sets teleosts apart from amphibians where the simple experimental approach of hypophysectomy / replacement therapy has yielded relatively unambiguous results.

1.3 The two-hormone hypothesis

Whilst there is considerable evidence that - at least in some teleost species - a pituitary melanophore-dispersing-hormone operates, the participation of a melanophore-concentrating hormone is a contentious issue. That two opposing hormones participate in physiological colour-change: the "two hormone hypothesis" - was first postulated by Hogben and Slome (1931) to explain the results of their experiments with Xenopus. This interpretation was criticised by Kent (1959b) and by Waring (1963) and refuted by the findings of Barker-Jorgensen (1962): a crucial tenet of the two hormone hypothesis was that hypophysectomised Xenopus

were not maximally pale, but this was shown to be false by Barker-Jørgensen who showed that the hypophysectomy technique used by Hogben and Slome almost certainly resulted in incomplete removal of the pituitary, and that totally hypophysectomised animals were indeed maximally pale.

In the meantime, the two-hormone hypothesis had been invoked by students of Hogben (Neill, 1940; Healey,1951) to explain background responses in teleosts. This interpretation has been criticised (for the cases of both amphibians and teleosts) by Kent (1959 b) who showed that some of the arguments for the two-hormone hypothesis were based on the invalid comparison of rates of adaptation to darkness with those of background reversal. Nevertheless, there remain a number of features of the background responses of teleosts which are difficult to reconcile in terms of one hormone. One such feature is the capacity of teleost pituitary extracts to induce pallor when injected into fellow teleosts (see Pickford and Atz 1957). Comparatively recently however, it has become apparent that the teleost pituitary is exceptionally rich in aminergic nerve-terminals - a feature of its peculiar mode of control by the hypothalamus (see Holmes and Ball 1974). Since teleost melanophores are highly sensitive to catecholamines, an obvious possibility (especially pertinent in view of the high doses necessary) is that the paling activity of pituitary extracts was

a pharmacological effect of catecholamines. Crucial therefore, to the concept of an MCH is the nature of the paling agent of teleost pituitary extracts. A number of workers have investigated this but the factor has never been isolated. Thus; Kent (1961) found that melanophore-dispersing and melanophore - concentrating activities (assayed using frog and minnow bioassays respectively) were inseparable by paper electrophoresis at a variety of pH's. He concluded that both activities were due to the same molecule, and that it was a peptide. Baker and Ball (1975) however, using the higher resolution technique of disk electrophoresis in polyacrylamide gels, were able to seperate multiple forms of MSH - none of which exactly co-migrated with MCH. These data are consistent with its putative peptidic nature and suggest that it is neither a type of MSH nor a catecholamine.

In their review of colour-change in lower vertebrates in 1968, Bradshaw and Waring re-evaluated the existing data, and concluded that hormonal control of the background responses of teleosts (as well as amphibia) was exerted soley by a dispersing hormone. Though they pointed out a number of inconsistencies with this interpretation, they felt that these did not warrant the postulation of a concentrating hormone in addition. Earlier, Waring had been a protagonist of the two-hormone hypothesis in teleosts and consequently

the 'melanophore-concentrating hormone' has been somewhat neglected since then.

In the eel Anguilla, where innervation is rudimentary, hypophysectomy severely impairs black background adaptation, full dispersion is restored by injection of pituitary extracts, and there is a clear case for the participation of MSH (Waring, 1963). The catfish Ictalurus behaves similarly, though nerves play a more active role in the paling process (Khokhar, 1970, 1971a,b). In neither case is an MCH thought to be involved in the paling process.

The evidence for an MCH comes from species where nerves have a dominant role in background adaptation and hypophysectomy has little or no effect thereon. To demonstrate the action of hormones therefore, and to preclude the participation of nerves, workers have applied a variety of de nervation techniques. Thus Healey (1948) has shown that minnows subjected to spinal section are still capable of background adaptation although its rate and extent are impaired by the operation. When spinally operated animals were hypophysectomised, even this limited ability was lost resulting in a permanently dark state. There is an obvious case here for an MCH. However, Grove (1969b) later showed that injection of the α-adrenergic antagonist Rogitine caused dispersion in minnows which were ostensibly pale due to circulating MCH which would

suggest that the pale state was due to circulating catecholamines rather than an MCH.

Baker and Ball (1975) have studied the effects of hypophysectomy on denervated melanophores of Poecilia. These workers used the fin-ray section technique of Parker (1948) to produce denervated caudal bands. Hypophysectomy alone (with intact innervation) reduced the extent of dispersion attainable on a black background suggesting a pituitary MSH might be involved in this response. (The extent of melanophore concentration attainable on a white background however, was unimpaired by hypophysectomy). In fish with denervated caudal bands, hypophysectomy delayed the onset of, and impaired the extent of white background adaptation. These workers noted the paling action of injected teleost pituitary extracts but in addition showed that extracts of the piuitary of Squalus (an elasmobranch) cause dispersion and - paradoxically - synthetic α-MSH caused slight concentration. They considered that these results supported the two-hormone hypothesis i.e. that pituitary control of melanophores was dual in character.

In evaluating the data which bears on the two-horomne hypothesis, it is important to consider some of the vagaries of the experimental methods used. Thus, Grove (1969a) compared the effects of spinal section and spinal-nerve section in Phoxinus. (The former is

section of the spinal cord anterior to the site of exit of the chromatic nerve fibres, the latter is section of the segmental spinal nerves distal to their exit from the segmental sympathetic ganglia). Grove concluded that the relatively rapid recovery of melanophore response in the denervated area resulting from the post-ganglionic section of segmental spinal nerves, was due to diffusion of neurotransmitter from neighbouring unaffected areas of skin. He also noted the long-term development of hypersensitivity to catecholamines in denervated melanophores (especially those which had been subject to post-ganglionic section).

Fujii and Novales (1969a) in their study of the nervous control of Fundulus melanophores used the caudal fin denervation technique of Parker (1948) and attributed paling in this region (in response to electrical stimulation) to diffusion of neurotransmitter as did Grove (1969a). They also noted (1969b) in support of this explanation that, typically, nerves terminated close to - but not on - melanophores. However, Baker and Ball (1975) have attributed paling of denervated caudal bands (of Poecilia) to MCH - since this process was impaired by hypophysectomy. It would seem therefore, that both circulating MCH and diffused neurotransmitter are involved.

In teleosts where innervation of the melanophores is highly developed - in contrast to the eel - there is

little evidence for the involvement of an MSH in physiological colour-change. In vitro studies have shown that in Oryzias latipes, α-MSH neither stimulates melanophores to disperse, nor does it accelerate the spontaneous dispersion which occurs in the absence of catecholamines (Negishi and Obika, 1980). Lack of response of teleost melanophores to mammalian hormones could be due to species differences in the structure of the hormones, but the close homology of mammalian and teleostean MSH's (Kawauchi and Muramoto, 1979; Kawauchi et al 1980a,b) would argue against this.

Recently, using a radioimmunoassay for α-MSH, Mr. T. Bowley of this laboratory has shown that plasma MSH titres in Salmo gairdneri are highest in fish adapted to black background. This is the first time circulating MSH has been measured in teleosts and is an important step in establishing hormonal status as it corroborates the PI PbH+ cell activation which occurs in this species. Considering this, in the context of the foregoing, it is more likely that (in fishes with dominant concentrating innervation) MSH is involved in morphological rather than physiological colour-change. (MSH could theoretically be involved in both but the evidence for a physiological role is lacking).

1.4 The source of MCH

Healey (1948) used partial hypophysectomy in spinal-sectioned *Phoxinus* and found that some of the anterior pituitary was required for maintenance of W response. Complete hypophysectomy abolished this response, and he concluded therefore that MCH was produced in the pars distalis (PD). By injecting homogenates of dissected pituitaries into *Phoxinus*, Kent (1959a) found - in contrast - that there was more MCH in the posterior half of the gland (i.e. the NIL).

Using an *in vitro* bioassay, Enami (1955) showed that in the oriental catfish, *Parasilurus asotus*, MCH was localised mainly in the posterior part of the anterior pituitary (the proximal pars distalis or PPD) as well as in the hypothalamus. He tentatively suggested that MCH was a type of neurosecretory hormone (i.e. a neurohypophysial hormone). If this were so it could explain the apparently discrepant observations of Healey and of Kent in *Phoxinus*.

A hypothalamic origin for MCH has also been suggested by Rance and Baker (1979) who found, with an *in vitro* assay, that the distribution of MCH between hypothalamus and pituitary of *Salmo gairdneri* varied according to background: whereas W-adapted fish had similar amounts at each site, in B-adapted fish more was found in the hypothalamus. They supposed that Kent's (1959a)

failure to detect MCH in the hypothalamus of Phoxinus may have been because his fish were W-adapted. They also found that the release of MSH from cultured NIL's was inhibited by cold, cycloheximide (a protein synthesis inhibitor), EGTA (a calcium ion chelating agent) and potassium ions, whereas that of MCH was erratic and not demonstrably affected by any of these treatments. These findings were cited in favour of an extra-pituitary site of biosynthesis (i.e. the hypothalamus) and it was suggested that the sporadic release of MCH might be due to the degeneration of MCH-containing axons.

1.5 MCH as a type of neurohypophysial hormone

The concept of MCH as a type of neurohypophysial hormone is attractive initially as it would explain the lack of cytophysiological evidence that either of the PI cells secretes a hormone during W-adaptation. However, there is no evidence that the neurohypophysis (NH) does so either, and a recent study of the effects of background on the cytology of the pituitary of Poecilia notes a lack of effect on the neurohypophsial elements (Ball and Batten, 1981). However, the secretion of a neurohypophysial hormone might not necessarily result in an observable change in the NH, although changes in features such as axon-terminal granule content and the amount of stainable neurosecretory

material present in the NH have been associated with osmoregulation (Lederis, 1963, 1964; Abraham, 1976), stress (Leatherland and Dodd, 1969) and with reproduction - see Perks (1969) for review.

The distribution of MCH between the hypothalamus and the pituitary described by Rance and Baker (1979) is contrary to that of the known neurohypophysial hormones where there is more in the pituitary due to storage in the axon-terminals of the NH. However, we know too little about the endocrinology of the neurohypophysial hormones of teleosts to regard this as necessarily inconsistent with the putative identity of MCH as a neurohypophysial hormone.

The apparent confinement of MCH to teleosts, or rather its absence from mammals, has meant that it has attracted little attention of late. However, the ever-increasing realization of the extent of homology which exists among hormones and related substances across phylogenetic boundaries puts MCH in a more attractive light - as a search for homologues in other species might reveal new hormones. Whether MCH is truly a hormone and whether it has homologues in other species are questions which await its purification, structural elucidation and further experiment.

There have been no published attempts at the outright

purification of MCH and this is the task addressed in this thesis.

CHAPTER 2

Materials and Methods

2.1 Fish

Rainbow trout (*Salmo gairdneri*) aged 1-2 yr (immature) were obtained from a local fish-farm (Alderley Trout Ltd., Alderley, Wooton Under-Edge). They were moderately dark on arrival at the laboratory and thereafter were kept in black or white tanks of aerated water (170 litres), without feeding, at 10°C with overhead illumination for a daily photoperiod of 16h.

2.2 Removal of pituitary glands and hypothalami

Fish were killed by decapitation and the roof of the cranium was sliced off. Grasping the spinal cord, the brain was lifted upwards and forwards (cutting nerves where necessary) far enough to expose the ventral pituitary; this resulted in severance of the pituitary stalk leaving the pituitary *in situ* and conveniently exposing the hypothalamus for dissection. The pituitary was then removed with a pair of fine forceps which were committed to this purpose to avoid cross-contamination of pituitary and hypothalamic extracts.

With the ventral surface of the brain uppermost, transverse cuts were made just posterior to the optic chiasma and just anterior to the saccus vasculosus. The ventral hypothalmus surrounding the

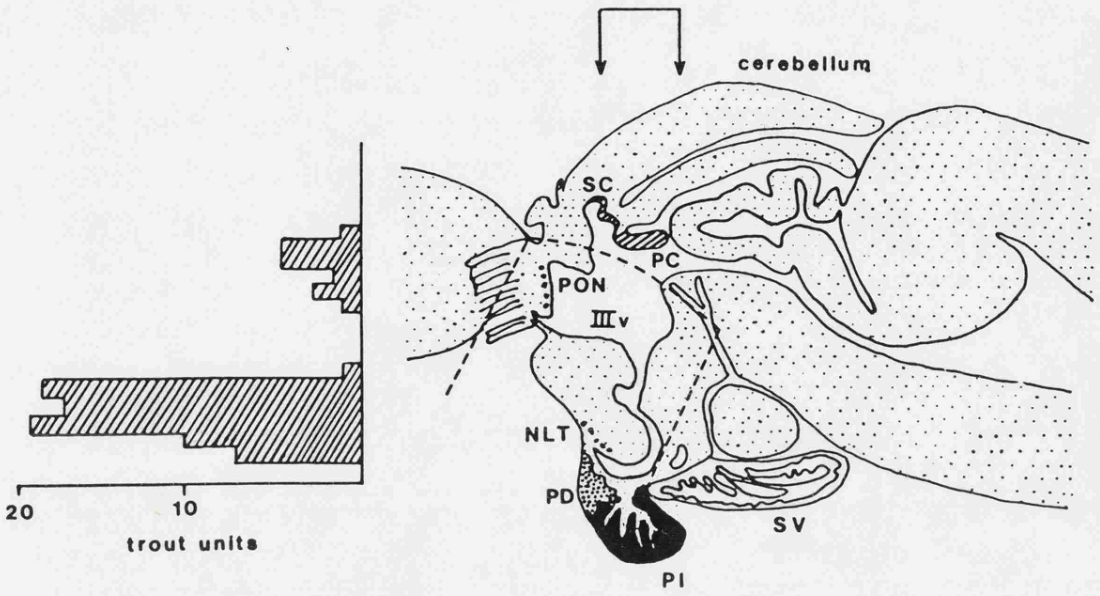


Fig. 1.

Diagram of the brain and pituitary gland of Salmo Gairdneri in saggital l.s. showing approximately the region of hypothalamus taken as source of MCH (broken line). The graph shows the MCH content of horizontal sections of brain determined after removal of the pituitary in the region denoted by the arrows. This region includes the posterior PON dorsally (not in this section) which extends around the lateral walls of the third ventricle, and also the NLT ventrally. The region excised probably includes, on average, the major bio-active peak of the ventral hypothalamus and also some of the upper peak. These data are unpublished observations of Drs. Rance and Baker of this laboratory. SC=sub-commisural organ, PC=posterior commisure, PON=preoptic nucleus, IIIv=third ventricle, NLT=nucleus lateralis tuberis, PD=and PI=pars distalis and intermedia of the pituitary, SV=saccus vasculosus.

third-ventricle between these cuts was then excised to a depth of about 3mm with a single horizontal cut with a pair of scissors. Fig. 1 shows the region excised by this procedure. Pituitaries and hypothalami were transferred to solid CO_2 immediately after removal. When sufficient had been collected, they were homogenised (hypothalmi first to avoid cross-contamination). With practise the pituitary and hypothalamus could be dissected and placed on solid CO_2 within 30 - 40s of the fish being alive.

2.3 Commercial pituitary powders

Because availability of fresh material was limited, commercial preparations of salmon (Onchorynchus keta) pituitaries were used for the purification of peptides. These were prepared by Syndel Laboratories of Vancouver, Canada, and supplied by Windsor Laboratories (formerly International Enzymes) of Slough, Bucks. At first, acetone-dried pituitaries were used, but because of the possibility that small peptides had been lost during preparation, a special batch of lyophilized pituitaries was obtained. These had been lyophilized directly from the frozen state without prior extraction. According to the suppliers, for each preparation dry weight / wet weight = 0.2, and 1g = 192 pituitaries.

2.4 Extraction procedures

Fresh tissue was homogenised either in 0.1M HCl or in 1M acetic-acid. An Ultra-Turrax homogeniser type TP18-10 was used at 20k Hz for 30s. Where smaller volumes (less than 5 ml) precluded the use of this homogeniser, tissue was sonicated at 20k Hz for 30s at 10μm amplitude using an MSE 100W sonicator with a 3mm terminal-diameter titanium probe. Where homogenisation was performed in boiling acetic-acid, fragments of frozen tissue were dropped directly into the boiling acid. (This was to minimise the risk of proteolysis which is serious when tissue is thawed, because of the rupture of lysosomal and other membranes caused by freezing). Pituitary homogenates were centrifuged at 2,400g for 20min and the pellet discarded. Hypothalamic homogenates treated in this way yielded a turbid supernatant, even when centrifuged at 9,000g. This was overcome by defatting the homogenate using chloroform / methanol according to Bligh and Dyer (1959), the resulting aqueous methanol phase was taken.

In the case of commercially prepared pituitary powders, 0.1M HCl gave poor solubilization, and therefore 1M acetic-acid (100ml per gram of powder) was used exclusively. Because, even with acetic-acid, the powders were more difficult to solubilize than fresh tissue, homogenization was for 5min in 30s episodes.

For chromatography, extracts were lyophilized and reconstituted in the running solvent.

2.5 Bioassay for MCH

The MCH bioassay was performed as outlined by Rance and Baker (1979). When a trout scale is placed in a physiological-salts solution containing MCH, the melanophores exhibit melanin-aggregation in a few minutes; both the rate and the extent depend on the MCH concentration of the solution. The assay is basically a titration whereby dilutions of a sample are tested in this way until one is found which will induce half-maximal melanophore-aggregation in 20 min. This dilution is known as the "threshold dilution". The activity of a sample in trout units (TU) is the reciprocal of this dilution X the volume of the sample expressed in tenths of a ml. For example:

if the threshold dilution = 1/100

and the sample volume = 0.2ml

then MCH activity =100/1 X 0.2ml = 20ml

=200TU

In practice the threshold dilution is rarely found and is estimated by interpolation between determinations either side of it. For each determination at least three scales were used.

Because of variation between batches of scales the trout unit is not an absolute measure and comparison can only be made within an assay. To compare between assays, a reference standard was prepared from the commercial acetone-dried salmon pituitary powder. An acetic-acid extract (unheated - see extraction procedures) was lyophilised in aliquots equivalent to 1mg of powder in 1.5ml conical polypropylene centrifuge tubes. These were then rigorously dessicated over P_2O_5 and NaOH and stored dessicated at -20°C. For assay a standard was reconstituted in 0.2ml of bioassay medium and the insoluble material centrifuged off at 9 000 g for 5min.

Because of the tendency of Tyrodes phsiological-salts solution to lose CO_2 and become alkaline on exposure to air, this was replaced by the HEPES-buffered medium described by Fujii and Miyashita (1975) for their experiments on guppy melanophores. Except where stated otherwise, this solution also contained 10^{-4}M Rogitine (Phentolamine mesylate, Ciba laboratories, Horsham, Sussex), 25mg l^{-1} phenol-red as pH indicator, and 0.1% polypep (Sigma,low-viscosity type). (Polypep was used as a carrier, its osmotic contribution was ignored; Rogitine is an α-adrenergic antagonist and was included to guard against catecholamine-induced concentration of melanophores). Scales scraped from the dorsal

surface of a freshly-killed black-adapted trout were immediately teased apart in this medium using dissecting needles under a binocular microscope. From these scales, a sub-population was then selected for uniformity of appearance with regard to melanin content, dispersion and size. These were then placed in a seperate petri-dish so as not to overlap one-another, with their epidermal side uppermost. These precautions were neccessary to minimize the spurious melanophore-concentration which occurs when scales are allowed to overlap. For assay, scales were taken at random from this sub-population.

For the detection of MCH activity, samples cannot usually be diluted sufficiently for the potentially toxic effects of solvents and the like to be ignored. For this reason, volatile buffers have been used throughout, and lyophilised samples reconstituted in bioassay medium for assay. For reconstitution, the amount of medium used was sufficient to buffer against acidic solutes as judged by the phenol-red indicator.

Because of the low peptide-content of fractions from HPLC, there was a danger that losses would occurr on lyophilisation of samples for bioassay. Such samples were therefore supplemented with 10µl of a 10mg ml^{-1} solution of polypep before lyophilisation.

Lyophilisation, dilutions and assays were all performed in 96-well, flat-bottomed, tissue-culture plates of 0.35ml well-capacity (N^{o} 76-001-05, Flow laboratories, Irvine, Scotland).

2.6 Measurement of melanophore area

Because of the subjective nature of the MCH assay, and the variation between melanophore responses of different scales, minimal interpretation has been used in the assessment of such responses: scales being described as positive(+), threshold (+/-) or negative (-). This forfeits information which might otherwise be obtained were an objective means of assessment available. For this purpose a Vickers M-85A flying-spot microdensitometer was evaluated for objective measurement of melanophore responses.

The area of individual melanophores was measured. The instrument scans the field of view measuring density continuously within an area prescribed by an adjustable circular mask. The result obtained is an integrated value of the density within this area. The stark contrast between melanophore and background enabled adjustment of a density-threshold facility such that densities less than a chosen value were counted as zero. In this way, the integrated density measurement was of the melanophore alone and did not require subtraction of background. The density-threshold

level was determined by measuring background density between concentrated melanophores - and increasing the threshold until the integrated density was reproducibly zero. The wavelength used was chosen as the λmax of the difference-spectrum between background and melanophore - determined by point measurements without the use of integration or threshold facilities.

Instrument settings were : wavelength, 650nm; beam intensity 35; objective lens, 25X; density threshold 0.2; and scan time 2s. Measurements were made with scales epidermal side uppermost and immobilised in a cavity slide with a cover-slip.

Treatment of scales with experimental solutions was as for the MCH assay. Catecholamine solutions were made immediately before use.

2.7 Bioassay for MSH

In the absence of a homologous assay system whereby teleost MSH's might be assayed on teleost melanophores, the Anolis assay of Burgers (1961) was used according to the detailed description of Tilders et al (1975). This assay is the same in principle as the MCH assay. Fragments of the skin of the lizard Anolis carolinensis are used to indicate the presence of MSH by a bright-green to brown colour change. Lizards were obtained from De Natuurvriend, Donkergaard, Utrecht, Netherlands.

The assay was standardised by comparison to α-MSH, results being expressed as μg-equivalents of α-MSH. The synthetic α-MSH standard used was the solution in 1% acetic-acid described under "radioimmunoassay of α-MSH". Not all assays were standardised in this way in which case results have been expressed in anolis units. The anolis unit is the same as the trout unit except that the basis of the unit is 1.0ml instead of 0.1ml.

Assays were performed in tissue-culture trays as for the MCH assay. Because of the greater dilutions involved, lyophilisation was generally unnecessary and samples were simply diluted. This was so, except when measuring sub-microgram amounts of MSH (i.e. when measuring MSH-contamination in highly purified preparations of MCH). In this case, where the MCH assay was to be conducted on the same fractions, reconstitution was performed in Anolis bioassay medium. This was necessary to keep Rogitine out of the Anolis assay because it prevents response to MSH.

2.8 Radioimmunoassay for α-MSH

The radioimmunoassay for α-MSH was developed by Mr. T. Bowley of this laboratory for the measurement of plasma MSH concentrations in trout. Its great sensitivity was not essential to this study and was sacrificed for increased range by increasing the concentrations of antiserum and radiolabelled MSH.

The assay is essentially a hapten radioimmunoassay: antisera were raised against a conjugate of synthetic α-MSH (Sigma) and bovine serum albumin formed with ethyl carbodiimide (Thody et al 1975). The conjugate was administered to rabbits by intramuscular injection as an emulsion in Freunds complete adjuvant (DIFCO). The particular antiserum used in this study is designated: R6FB.

Radioiodination was performed using "chloramine T" according to Greenwood et al (1963); 1mCi of ^{125}I was used to iodinate 2μg of peptide. After iodination the mixture was diluted with 300μl of assay buffer and passed through a column of ODS-Spherisorb (10μm grade, Phase seperations, Queensferry, Scotland). The adsorbed peptide was eluted stepwise and the fraction eluting in 44% v/v methanol/1% TFA was taken. Radioiodinated α-MSH (^{125}I-α-MSH) stored in this solution at -20°C was used for up to 3 weeks. Synthetic α-MSH (Sigma) was used as standard, amounts of 20μg—200μg were weighed accurately on a Cahn electrobalance and dissolved in 1% v/v acetic acid to a concentration of 200μg ml^{-1}. This solution was diluted in assay buffer to 2μg ml^{-1} and stored in 0.5ml lots in 1.5ml polypropylene centrifuge tubes at -20°C. Assay buffer consisted of 0.05M sodium phosphate pH 7.6 containing 5% v/v inactivated horse-serum (Wellcome N° 5) and 0.02% w/v ethyl mercuric thiosalicylate

Table 2

Crossreactivity of α-MSH antiserum R6FB with a variety of part-sequences of hACTH

Peptide	% cross reactivity at 50% displacement	slope of competition curve
α-MSH	100	parallel
desacetyl-α-MSH (1-13 NH_2)	70	
1-16	0.25	
1-16 NH_2	0.23	
1-10	<0.07	non-parallel
1-24		
4-10		

This data is unpublished work of Mr. T. Bowley of this laboratory. Cross-reactivity is expressed relative to that of α-MSH in molar terms. Synthetic peptides were a gift from Dr. W. Rittel of Ciba Laboratories, Basel, Switzerland. This antiserum appears to recognise the -Lys-Pro-Val-NH_2 sequence at the C-terminal of α-MSH.

("Thimerosal", Koch-Light). This solution was used as diluent for all samples and standards.

Incubation was at 4°C overnight in a volume of 400µl containing 40 000cpm ^{125}I-α-MSH and antiserum at a final dilution of 1/20 000. Bound and free label were then separated by adding 400µl of suspension of dextran-coated charcoal. After 15min, the suspension was centrifuged (4 600g, 15min, 4°C) and the free counts present in the charcoal were determined after aspiration of the supernatant. The charcoal suspension contained: assay buffer, 70ml; inactivated horse-serum, 30ml; charcoal (Sigma C/5260) 0.5g and dextran (Pharmacia T70), 0.125g. Charcoal was activated before use by heating to 200°C overnight. The suspension was stirred at 4°C for 30min before use.

2.9 Radioimmunoassay for ACTH

The procedure for radioimmunoassay of ACTH was substantially the same as that for α-MSH except for the following:

The antiserum used was Wellcome's anti-hACTH-serum RD 05 lot N° K7653 (Wellcome, Beckenham, Kent). For standardization, Wellcome hACTH standard N°140872 (2.43ng per vial) was used. The procedure used two separate incubations: the first, in 200µl of assay

buffer contained antibody (at 1/20 000 dilution) and sample, and was for 72h at 4°C; after addition of ^{125}IpACTH (9 800 counts in 100µl) the mixture was incubated for a further 6-8h at 4°C and reaction was terminated by addition of dextran-coated charcoal as described by Rees et al (1971).

For radioiodination, porcine ACTH was used, this was supplied by the National Institute of Biological Standards, Hampstead, London code N° 69/22R. This preparation has been calibrated by NIBS in terms of the World Health Organization's third international standard pACTH, as containing 5 IU per vial. The contents of a vial were dissolved in 125µl of 0.005M HCl and stored in 10µl lots at -20°C. For iodination 5µl (containing 2µg) was taken. The iodinated peptide was purified by adsorption onto QUSO G-32 glass granules as described by Rees et al (1971).

Assay buffer was 0.05M sodium phosphate buffer pH7.4 containing 2.5mg ml^{-1} bovine serum albumin (Sigma RIA grade) and 0.5% v/v 2-mercaptoethanol. (The mercaptoethanol is to minimise "incubation damage" which is a problem with ACTH (Yalow and Berson 1976)).

2.10 ODS-extraction

Peptides in aqueous solution will adsorb strongly to hydrophobic surfaces such as octadecyl silanised silica

(ODS-silica), from which they can be displaced by water-miscible organic solvents such as methanol and acetonitrile. This is the basis of the peptide-extraction technique of Kelly et al (1978). Adaptations of this method have been used to extract peptides from crude extracts and to desalt phosphate-containing fractions from HPLC system 2.

For crude extracts in HCl or acetic acid, ODS-Porasil A was used. Porasil A (Waters Associates, Northwich, Cheshire) was reacted with octadecyl-trichlorosilane (ODS Koch-Light, Colnbrook, Berks.) as described by Bennet et al (1977). Columns were prepared by packing Porasil into 1 or 5ml Plastipak syringe barrels between discs of porous teflon (Waters). Extracts were passed repeatedly through the column (nine times in all), after which the column was washed with 1% TFA (to remove non-adsorbed substances). Adsorbed peptides were then eluted with 80% methanol/ 1% TFA. Loads up to 50mg of pituitary powder per ml of ODS-Porasil were applied and columns were discarded after use.

To desalt phosphate-containing HPLC fractions, 0.25ml columns of ODS-Spherisorb were used. To allow peptides to adsorb to the ODS, the acetonitrile concentration was reduced by diluting the fractions fivefold with deionised water. After a single passage of the sample through the column, it was washed with 0.5ml of deionised

water and eluted with 2 X 0.5ml of 80% methanol/ 1% TFA. After a 2ml water-wash, columns were re-used up to forty times.

2.11 Gel-exclusion chromatography

Gel-exclusion chromatography (gel filtration) was performed using Bio-Gel P2 200-400 mesh (Bio-Rad Laboratories, Watford) and Sephadex G-25 fine (supplied by Sigma, Poole, Dorset). Columns were eluted with and samples were applied in - 1M acetic acid (pH 2.4). The absorbance of the effluent was monitored continuously at 280nm using an ISCO model UA 5 spectrophotometer with a type 6 optical unit fitted with 0.5cm path-length flowcells (supplied by Fisons of Loughborough).

2.12 Two-dimensional cellulose thin-layer electrophoresis/chromatography

For analytical purposes this technique was carried out according to Heiland et al (1976) using Merck glass-backed cellulose TLC plates of 0.1mm layer thickness (product number 5716, supplied by BDH, Poole). Where larger sample loads were used the two methods were performed separately and sample was applied to the dry plate as a 5cm band. In electrophoresis, the sample was applied first (methyl-green was applied in a seperate spot as a reference) and the plate was sprayed with solvent. The solvent-system for electrophoresis was: pyridine, glacial acetic acid, acetone and water

in the ratios 1:2:8:40 v/v respsctively pH4.4. That for chromatography was: n-butanol, acetic acid, pyridine, and water in the ratios 15:3:10:12 v/v respectively. Plates were air dried and the seperated peptides were visualised by spraying with a 0.01% w/v solution of fluorescamine (Sigma) in acetone/ pyridine 9:1 v/v. Where bioassays were to be performed the plate was scraped in 5mm bands and peptides were eluted with 3 X100µl of 50% v/v acetic acid. This was achieved by transferring the resulting powder to a syringe-barrel tightly plugged with porous teflon, and washing the powder by successive vortexing and centrifugation (at 2 000g for 2mins) with the acetic acid (Fig 2).

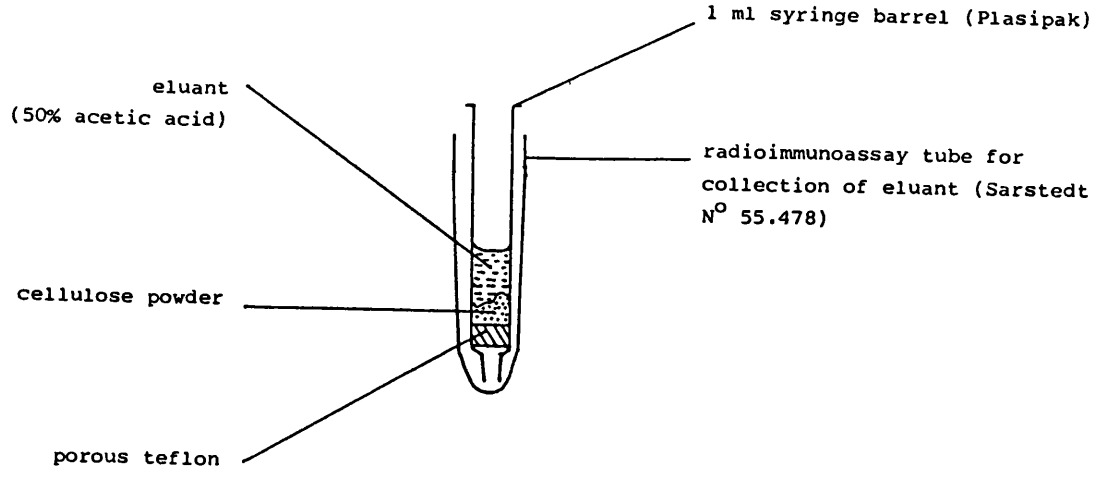


Fig 2 Apparatus for eluting TLC fractions shown before centrifugation.

2.13 Ion-exchange chromatography

The cation-exchanger SP-Sephadex C-25 (sulpho-propyl Sephadex, supplied by Sigma) was converted to the ammonium form by repeated washing and decantation in 1M ammonium acetate pH7. After equilibration with 0.05M ammonium acetate pH4.8 containing 20% v/v methanol (starting buffer), a column was packed to give a bed of dimensions: 30 X 2.5cm. Samples were prepared by dissolving lyophilized extracts in starting buffer and centrifuging off that which remained insoluble at 90 000g for 30min. The supernatant was applied to the column followed by 200ml of starting buffer to elute those substances not bound to the exchanger under these conditions. This was followed by an 800ml gradient of ammonium acetate of increasing concentration and pH at constant methanol concentration (20% v/v). The gradient was from 0.05M pH4.8 to 1.0M pH7.0 and was linear with respect to concentration. Eluate absorbance was monitored as for gel-filtration.

2.14 High-performance liquid chroamtography (HPLC)

Two HPLC systems were used: the first was that of Bennet *et al* (1977) and used prepacked analytical-scale columns (250 X 4.6 mm) or semi-preparative scale columns packed in the laboratory (300 X 8 mm) containing Partisil-10-ODS (irregularly shaped octadecyl-silanised silica of 10μm particle-

size supplied by Whatman,Maidstone, Kent). Columns were slurry packed by upward flow from methanol. HPLC accessories were obtained from HETP of Macclesfield, Cheshire. Columns were eluted with linear gradients of increasing methanol concentration in 1% v/v TFA generated at low pressure using syphon-linked beakers,and were delivered by a single Milton-Roy instrument mini-pump. The absorbance of the eluate was monitored continuously at 280nm using an LKB Uvicord II spectrophotometer with a 2cm path-length micro flow cell. As the solvents used were completely volatile, peptides could be recovered by evaporation under reduced pressure. Samples dissolved in the primary solvent (1% TFA) were applied to the column through the pump.

The second HPLC system was a purpose built apparatus manufactured by Altex, supplied by Anachem of Luton. Gradient formation was microprocessor controlled via models 100 and 110 solvent metering pumps. U.V. detection was at 225nm using a Pye-Unicam variable wavelength spectrophotometer with a 10 X 1mm, 8µl flowcell. Tripartite linear gradients of increasing acetonitrile concentration in 0.2M phosphate buffer pH 2.1 were applied to laboratory-packed analytical scale columns (150 X 5mm) of Nucleosil-5-ODS (Macherey-Nagel, Duren, G.F.R.) or Hypersil-ODS (Shandon, Runcorn, Cheshire). The buffer was 0.1MNaH_2PO_4 brought to pH 2.1 with

H_3PO_4 (total phosphate concentration 0.2M). The secondary solvent was 60% v/v acetonitrile in this buffer. This method is basically that of O'Hare and Nice (1979). Samples were applied through a high-pressure injection- valve fitted with a 2ml sample loop. The greater U.V. transparency of the acetonitrile/phosphate system enabled the use of a shorter wavelength for the detection of peptides regardless of whether they contained tryptophan.

2.15 Fluorometric determination of tryptophan content of peptides

Detection and measurement of the tryptophan fluorophor was achieved by obtaining emission spectra of peptide samples excited at 280nm in 80% methanol/1% TFA. Slit widths were : excitation, 2mm; emission, 0.5mm - this was a useful compromise between sensitivity and resolution.

All fluorescence measurements were performed in an Aminco SPF 125 spectrophotometer with a 1cm quartz cuvette containing 1.0ml of sample.

2.16 Amino-acid analysis

Amino-acid analysis was performed on acid hydrolysates of peptide samples using an amino-acid autoanalyser and also by a highly sensitive manual isotopic method using dansyl chloride. Hydrolyses were performed with

100µl of Aristar 6M HCl (BDH, Poole), 0.5ml/l 2-mercapto-ethanol (Sigma type I) in sealed, evacuated Pyrex tubes (75 X 10mm, No 1622 302) at 105^{o} for 16h. Sometimes 3M mercaptoethane sulphonic acid (MESNA, Pierce Sequanal grade) was used for the estimation of tryptophan as well as all of the other amino-acids. After hydrolysis for 24h in 100µl of MESNA, samples were neutralised to pH≈1.2 and diluted by addition of 1.4ml of 0.15 M NaOH. 300µl samples of the resulting solution were pumped directly onto the autoanalyser column. (This is a micro-version of the method of Penke et al, 1974). All glassware was cleaned by conventional means followed by "ignition cleaning" by treatment at 500^{o}C overnight in a muffle furnace. Care was taken to avoid contamination from fingerprints and dust.

The amino-acid autoanalyser was a Chromospek by Rank Hilger of Margate, Kent. Calibration of both methods was performed with Calbiochem's AA5 amino-acid mixed standard (Calbiochem, Bishops Stortford, Herts). The manual method used was that of Brown and Perham (1973). This technique uses ^{14}C-amino-acid internal standards and ^{3}H-dansyl chloride (^{3}H-Dns Cl). Samples of ^{14}C amino-acid mixture and of peptide hydrolysate are mixed and allowed to react with ^{3}H -DnsCl. The resulting Dns-derivatives are separated by two-dimensional TLC on polyamide plates, and the ^{3}H/^{14}C ratio for

each is then then determined by liquid scintillation counting. For a given Dns-amino-acid, the $^{3}H/^{14}C$ ratio is proportional to the amount of unlabelled amino-acid present in the hydrolysate. The method is first calibrated using the AA5 mixture and thereafter standard-curves are used to convert $^{3}H/^{14}C$ ratios to pmoles of amino-acid.

Radiochemicals were obtained from the Radiochemical Centre, Amersham. Supplementation of the ^{14}C amino-acid standard mixture with ^{14}C-histidine was unnecessary as this is now included by the manufacturers. The commercially available scintillant Unisolve 1 (BDH) was found to be a suitable substitute for that used by the original authors. Channels were set on the scintillation counter for use with this scintillant such that effectively no ^{3}H counts (<1%) appeared in the ^{14}C channel, and 35% of ^{14}C counts appeared in the ^{3}H channel. The fragments of TLC plate were not removed from the vials before counting but this did not interfere. Schleicher and Schull two-sided micropolyamide TLC plates (F1700) were obtained from Pierce and Warriner (UK) of Chester.

2.17 MCH stability experiments

An extract of 250mg of acetone-dried pituitary powder was prepared in 5ml of 1M acetic-acid at room temperature. Lyophilised or ODS-extracted samples of this extract

were used for the following treatments.

a) TPCK-Trypsin digestion

A lyophilised 1ml sample of the acetic acid extract was reconstituted in 1ml of bioassay saline. To 100µl aliquots of this solution, 900µl samples of each of the following fresh solutions was added:

(i) TPCK-trypsin 200µg ml^{-1} (trypsin digest)

(ii) TPCK-trypsin 200µg ml^{-1} containing 500µg ml^{-1} ovomucoid trypsin inhibitor (control for the non-enzymic effects of added trypsin).

(iii) Ovomucoid trypsin inhibitor 500µg ml^{-1} (control for the non-specific effects of added trypsin inhibitor)

(iv) Saline alone (control for autodigestion by endogenous protease).

After 2h at 37°C reaction was stopped by addition of trypsin inhibitor (1ml containing 500µg). Trypsin was Sigma type X11 N° T 2884; to ensure freedom from chymotryptic activity it was first treated with L-1-tosylamide-2-phenylethyl chloromethyl ketone (TPCK) according to Carpenter (1967). Trypsin inhibitor was Sigma type III-O N° T 2011 ovomucoid trypsin inhibitor.

b) Pepsin digestion

To 100µl of acetic acid extract, 400µl of a fresh

solution of pepsin (100µg ml^{-1} in 1.5% v/v formic acid) was added. As a control, half of this solution was immediately heated to 90°C for 5min to denature the enzyme; the other half was incubated at 37°C for 2h and then denatured. Both samples were then frozen, lyophilised, and reconstituted in bioassay medium. Pepsin (EC 3.4.23.1) was Sigma N°P7012.

c) Cyanogen bromide treatment

To 100µl of crude extract, an equal volume of a 5mg ml^{-1} solution of cyanogen bromide in 90% v/v formic acid was added. Samples were incubated at 40°C for 2h. Non-peptide reaction products (except Br^-) were removed by lyophilisation. A reagent blank was prepared using 100µl of 1M acetic acid instead of extract. After lyophilisation, 100µl of extract was added to the blank which was again lyophilised; this served as a reagent-blank control for the potentially toxic effects of non-volatile reaction-products.

d) Hydrogen peroxide oxidation

The procedure of Neumann (1972) for the residue-specific oxidation of the methionine residues of ribonuclease was adapted as follows. For this treatment, the sample must be free of halide ions and organic acids because of their tendency to form free atoms and organic peracids in the presence of hydrogen peroxide. A 1ml

sample of the acetic acid extract was therefore extracted by repeated passage through a 2ml column of ODS-Porasil packed in a 5ml syringe barrel, after washing with water, the adsorbed peptides were eluted with 80% v/v methanol (without TFA). The dried residue of the aqueous methanol eluate was reconstituted in 2ml of distilled water and half of the sample was oxidised by addition of 30µl of 30% v/v hydrogen peroxide and incubated at 37°C for 2h (the other half was incubated without hydrogen peroxide as control). Remaining peroxide was removed before bioassay by dropwise addition to 400µl of a solution of catalase (Sigma, thymol-free N°C40) at 1mg ml^{-1} in 0.01M phosphate buffer pH7.0. The MCH activity of the untreated half was compared to that of the oxidised sample and to an oxidised sample which had been reduced with thioglycollic acid.

e) Thioglycollic acid reduction

To 100µl samples of crude acetic acid extract and H_2O_2-oxidised extract, 400µl of a 0.5% v/v solution of thioglycollic acid in distilled water was added. Tubes were flushed with nitrogen, sealed, and incubated at 50°C for 5h.

f) Mild alkali treatment

A lyophilised sample of 100µl of crude extract was

reconstituted in 500µl of 1M ammonium acetate buffer pH 10.5. This was incubated at 37°C for 2h. Reaction was stopped by freezing and lyophilisation. This procedure was designed to test stability to alkali without causing racemization.

2.18 Reagents and solvents

Except where specified otherwise reagents and solvents were analytical reagent grade. For HPLC, methanol and acetonitrile were HPLC grade (Rathburn Chemicals, Walkerburn) or Spectrograde (Fisons, Loughborough) and aqueous solvents were filtered by passage through a 50 X 8mm column of Partisil 10 ODS. For spectrofluorometry, acetone and methanol were spectrograde and aqueous solvents were filtered using Millipore 0.2µm filters.

2.19 Fast-atom bombardment mass-spectrometry of peptides

Mass-spectral analysis of peptides was performed by Ms. C. V. Bradley of the University Chemical Laboratory, Cambridge. Mass-spectra were obtained on a Kratos MS 50 instrument operated at accelerating voltages from 5 - 8kV. Samples were introduced to the ion-source on a copper probe tip via a conventional vacuum lock. Peptide samples dissolved in a small quantity of glycerol were applied to the probe-tip as a thin film. The area of the tip was 0.1 - 0.25cm^2 and typically 10 - 50% of this was covered with the glycerol

solution.

Ions from the sample were obtained in the gaseous phase by bombardment of the glycerol solution with Ar atoms at 4 - 6 keV at a pressure of about 10^{-5} torr. These atoms were obtained by charge-exchange with 4 - 6 keV Ar^{+} ions, using a standard fast-atom bombardment source and associated fast-atom gun (Kratos, Urmston, Manchester). Spectra were scanned from high to low mass at rates from 100 - 1000 s per decade.

CHAPTER 3

RESULTS

3.1 The effect of various chemical and enzymatic treatments on the activity of MCH

Introduction

A variety of factors were investigated for their effect on the activity of MCH in an attempt to gain information on its chemical nature. For these experiments a crude unheated extract of 250mg of acetone-dried pituitary powder was used. Samples of this were taken for each treatment - the activity of experimental samples being measured relative to appropriate reagent blank controls (see section 2.17).

a) Trypsin

TPCK-trypsin caused a drastic reduction in MCH activity (Table 3). This effect was prevented by excess trypsin-inhibitor and was therefore due to the enzymic action of trypsin and was not a non-specific effect of added protein.

Most preparations of trypsin contain a small amount of chymotrypsin, and unless this is removed, the action of chymotrypsin can be erroneously attributed to trypsin. In this way substance P was once falsely identified as being trypsin-sensitive (see Leeman et al 1977). For this reason TPCK treated trypsin was used.

Table 3 The effect of various chemical treatments on the activity of MCH

treatment	activity relative to control*
trypsin	1/128
pepsin	16
cyanogen bromide	1/8
hydrogen-peroxide oxidation	2
thioglycollate reduction	1
hydrogen peroxide oxidation followed by thioglycollate reduction	1/2
mild alkali (pH 10.5)	1

* In each case, appropriate reagent-blank controls wereincubated in parallel with the experimental samples (see section 2.17). Two-fold dilution series of experimental and control samples were compared within the same assay.

(TPCK is an active-site directed irreversible inhibitor of chymotrypsin; TPCK-trypsin was prepared according to Carpenter (1967)).

Since trypsin is very specific for peptide bonds on the C-terminal side of Arg or Lys residues, we can infer that MCH is a peptide containing either or both of these residues. Furthermore, we know that if MCH contains only a single Lys or Arg, this can not be situated at the C-terminal. The existence of Arg and /or Lys in the peptide may explain its cationic behaviour during electrophoresis and ion-exchange chromatography.

b) Pepsin

Although pepsin prefers to cleave peptide bonds on the C-terminal side of aromatic residues (Trp, Phe, Tyr), its specificity is rather broad and it will cleave most peptides to small oligopeptides eventually. It was unexpected therefore to find that treatment with pepsin caused a 16-fold enhancement in MCH activity (Table 3). Perhaps this represents liberation of MCH from a precursor in the pituitary extract by limited proteolysis. Conversely, this could be caused by destruction of an inhibitor. However, although there appears to be an inhibitor in the hypothalamus - this is not so for the pituitary (section 3.9).

c) Cyanogen bromide

Treatment with cyanogen bromide will cleave peptide bonds on the C-terminal side of methionine residues. This was found to reduce MCH activity to 1/8 of the control value (Table 3). The great excess of this reagent used (in molar terms) would suggest that partial inactivation was not due to incomplete progress of the cleavage reaction. These results would suggest that MCH undergoes cleavage with cyanogen bromide to form products of lower biological activity than the intact peptide, which would infer the existence of a methionine residue in MCH.

d) Oxidation with hydrogen peroxide

Oxidation with hydrogen peroxide, which was designed to oxidise methionine residues to the sulphoxides (section 2.17d) was found to cause about a 2-fold enhancement in MCH activity (Table 3). The significance of such a small difference is questionable in view of the poor precision of the MCH bioassay. However, that this finding is significant is indicated by the observation that subsequent reduction with thioglycollate caused a fourfold reduction in MCH activity, whilst thioglycollate reduction of non-oxidised samples was without effect.

Generally, methionine sulphoxide derivatives of biologically active peptides are less active than

their native counterparts and it would appear that MCH is exceptional in this respect.

e) Reduction with thioglycollate

Reduction with thioglycollate had no apparent effect on the activity of MCH. Since this treatment will break disulphide bridges yielding separate cysteine residues; either MCH does not contain a disulphide bridge, or if it does - then this is not essential to its biological activity.

f) Oxidation with hydrogenperoxide followed by reduction with thioglycollate

Since treatment with thioglycollate will reverse the effect of peroxide-oxidation on methionine residues (i.e. will regenerate methionine side-chains from methionine-sulphoxide side-chains); were the effect of peroxide-oxidation on MCH due to oxidation of methionine, then this should be reversed by subsequent reduction with thioglycollate. To investigate this, an aliquot of the sample which had been oxidised with hydrogen peroxide was reduced with thioglycollate. The net effect of these two treatments was to reduce MCH activity to about half that of the control value (Table 3).

Because of the dubious significance of such a small difference, this observation is consistent with the hypothesis that the peroxide induced enhancement in the biological activity of MCH was via oxidation of methionine residues.

g) Treatment with mild alkali (pH 10.5)

Rudman et al (1979) have shown that mild alkali (pH 10.5) will remove the O-acetyl group of diacetyl-serine-α-MSH converting it to α-MSH. To see if MCH contained an alkali-labile group such as this, its stability was tested in 1M ammonium acetate buffer pH 10.5. After incubation at 37°C for 2h there was no detectable change in MCH activity.

3.2 Evaluation of chromatographic methods for the purification of MCH

Introduction

The original aim of this project was the isolation of MCH. This has not previously been attempted and it was necessary therefore to evaluate methods which might be used preparatively to isolate MCH. The main criterion used to evaluate methods was their ability to seperate MCH from MSH. This was considered important since Kent (1961) had hypothesized that teleost pituitaries contained a single factor which was responsible for both biological activities.

a) Gel-filtration

When extracts of fresh trout-pituitaries were subjected to gel-filtration on Biogel P2, peaks of MCH and MSH bioactivity were found which were sufficiently separate to indicate that the factors responsible for concentration of trout melanophores and dispersion of Anolis melanophores were different (Fig 3). Since both eluted after the void volume (Vo), this suggested that they were less than 1800 daltons (the nominal exclusion-limit of this gel). Fig 3 also shows clearly that MCH is not a catecholamine since its elution position differs greatly from that of dopamine.

b) HPLC of crude extracts

HPLC of extracts of fresh pituitaries resolved MCH into two peaks but only achieved partial separation from MSH which also was resolved into multiple peaks (Fig 4). The existence of two types of MCH is confirmed by the results of ion-exchange chromatography (section 3.3).

c) ODS-extraction

Since MCH was reversibly retained by ODS-silica during HPLC, it was considered that ODS-extraction (Kelly et al, 1978) might be used as a first step in the purification of MCH. Indeed, Kelly et al have shown that peptides can be extracted in this way from acid

extracts in high yield. Because MCH is difficult to quantify (see section 3.5), to monitor recovery ^{125}I-α-MSH was used as an internal standard; this was justified by its similar elution position to MCH in HPLC on ODS-silica. Fig 5 shows how repeated passage of an acid extract of pituitary powder through a column of ODS-Porasil resulted in progressive accumulation of label by the column. Altogether 70% of the label was accumulated by the column, of which 99% was afterwards eluted in a single wash with 0.5ml of 80% methanol / 1% TFA. The load used was deliberately high (an extract of 10mg of pituitary powder per 0.4ml bed-volume) and failure of the label to adsorb readily or completely to the ODS-Porasil may indicate that its capacity was exceeded. Later it was realized that at least gram quantities of pituitary powder would be needed to purify MCH. This would require amounts of ODS-Porasil so large as to be impracticable at this stage of purification and the procedure was not adopted as the first purification step.

d) Thin-layer methods

Although MCH can be recovered from PAGE on an analytical scale, this is not a good preparative method. In contrast, thin-layer methods are often used for peptide purification and recovery of peptides is facilitated by the use of volatile buffers.

During thin-layer electrophoresis and thin-layer chromatography MCH migrated as a single peak (Fig 6). Having established that MCH could be recovered from these techniques, they were then evaluated for their ability to separate MSH and MCH. Fig 7 shows the two methods performed successively on the ODS-Porasil extract of Fig 5. Clearly these methods were ineffective at separating MCH and MSH.

Baker and Ball (1975) were able to separate MCH from the α-MSH's of trout pituitary by polyacrylamide gel electrophoresis (PAGE). The greater effectiveness of PAGE was probably attributable to the molecular sieving properties of the gel since the pH of both thin-layer electrophoresis and PAGE were similar (pH 4.4 and pH range 3.8 - 4.4 respectively).

The recovery of ^{125}I-α-MSH internal standard after ODS-extraction and thin-layer electrophoresis / chromatography is given in Table 4. Recovery of this label was particularly low from TLC. This was probably because at this stage of purification the label was essentially free of carrier peptides, since visualization with fluorescamine showed no peptide spots in the region of the chromatogram from which radiolabel was recovered. i.e. These results do not necessarily indicate that recovery from thin-layer chromatography is inherently inferior to that from thin-layer electrophoresis.

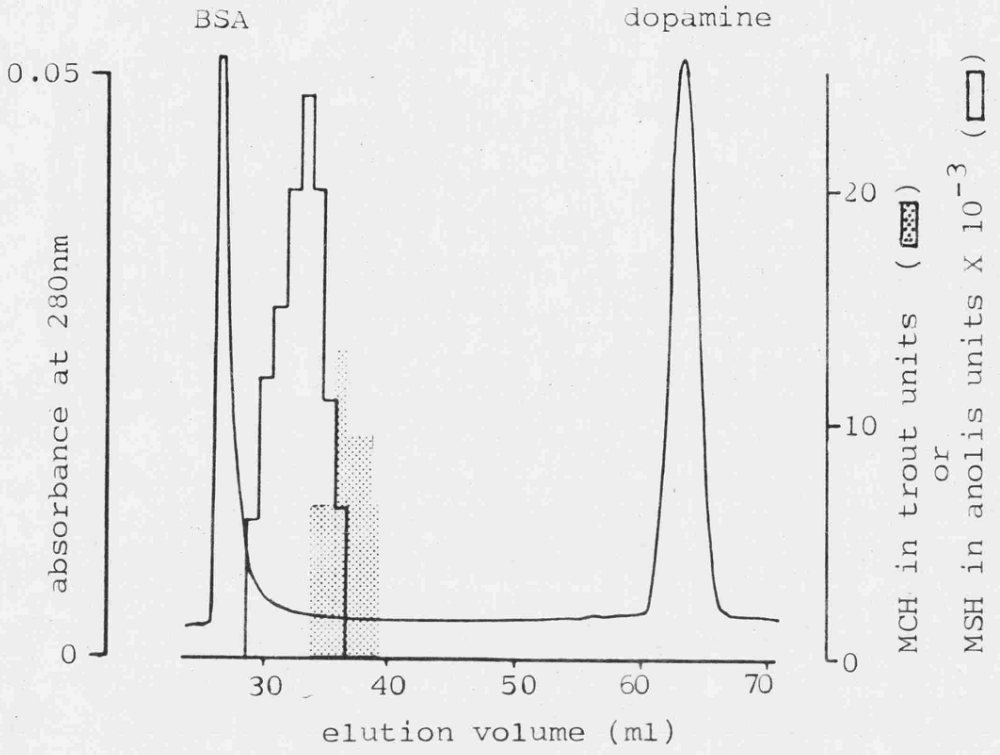


Fig. 3 Gel-filtration of an ODS-Porasil extract of trout pituitaries on Bio-gel P2 (exclusion limit 1800 daltons). An extract of 70 pituitaries was prepared by sonication in 3.0ml of 0.1M HCl. After centrifugation the supernatant was passed through a 0.4ml column of ODS Porasil. Adsorbed solutes were eluted with 0.5ml of 80% methanol / 1% TFA and this solution was evaporated to dryness under partial vacuum. The residue was reconstituted in 300 µl of 1M acetic acid and applied to a 70 X 1.1 cm column of Bio-gel P2 eluted at 7 ml min^{-1} in the same solvent. Fractions of 1.0 ml were collected and bioassayed for MCH and MSH. The elution positions of BSA and dopamine - denoted by the absorbance trace - were determined in a separate run on the same column.

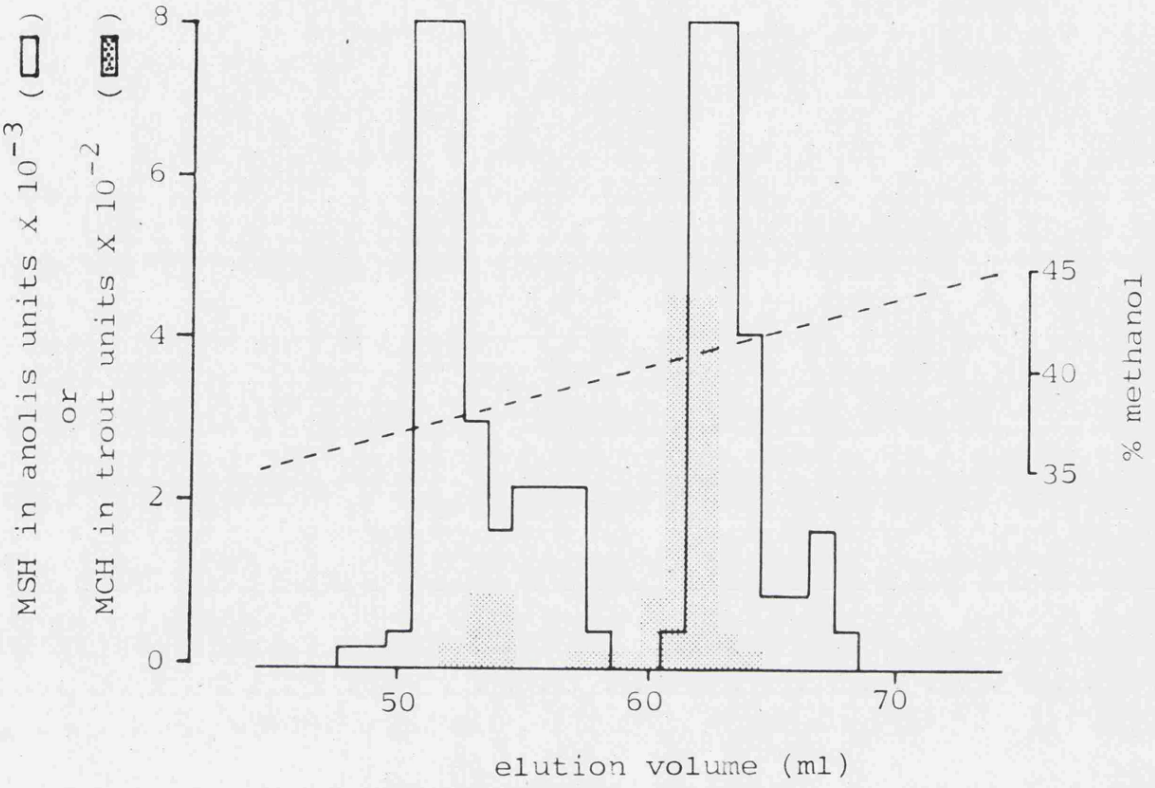


Fig. 4 HPLC (system 1) of an ODS-Porasil extract of 70 fresh trout pituitaries. The extract was prepared as for Fig. 3. The dried residue was reconstituted in 2ml of 1% TFA and applied to a 250 X 4 mm column of Partisil-10-ODS operated at a flowrate of 1.0 ml min^{-1}. This was followed by a simple linear 178ml gradient from 20-80% methanol. Only the region of the chromatogram where bioactivity eluted is shown.

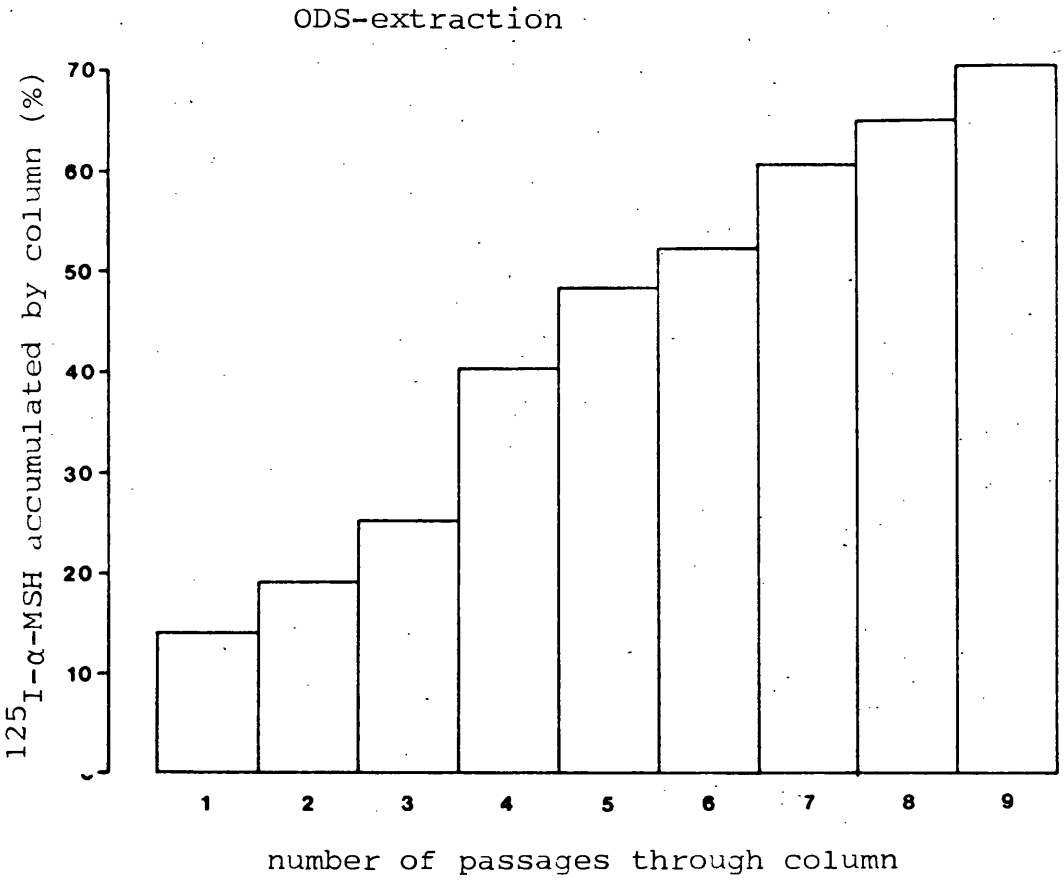


Fig 5 An extract of 20mg of acetone-dried pituitary powder was prepared in 1.0ml of 0.1M HCl by sonication at room-temperature. After centrifugation, 12 000 cpm. of ^{125}I-α-MSH (about 25pg) was added to the supernatant which was passed repeatedly through a column of ODS-Porasil (0.4ml bed-volume). At each step the amount of radiolabel which had adsorbed to the ODS-Porasil was determined by counting both the eluate and the column.

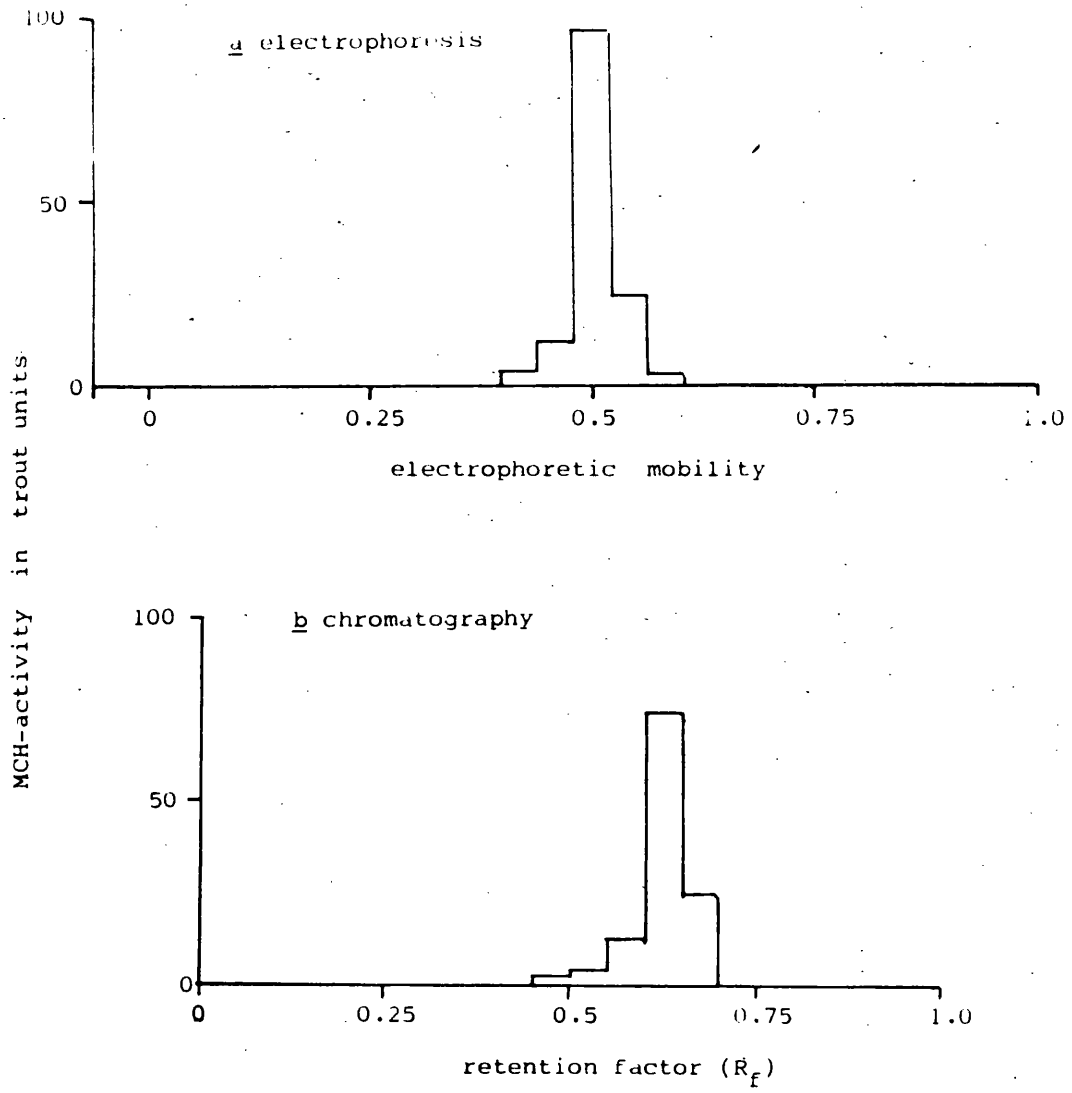
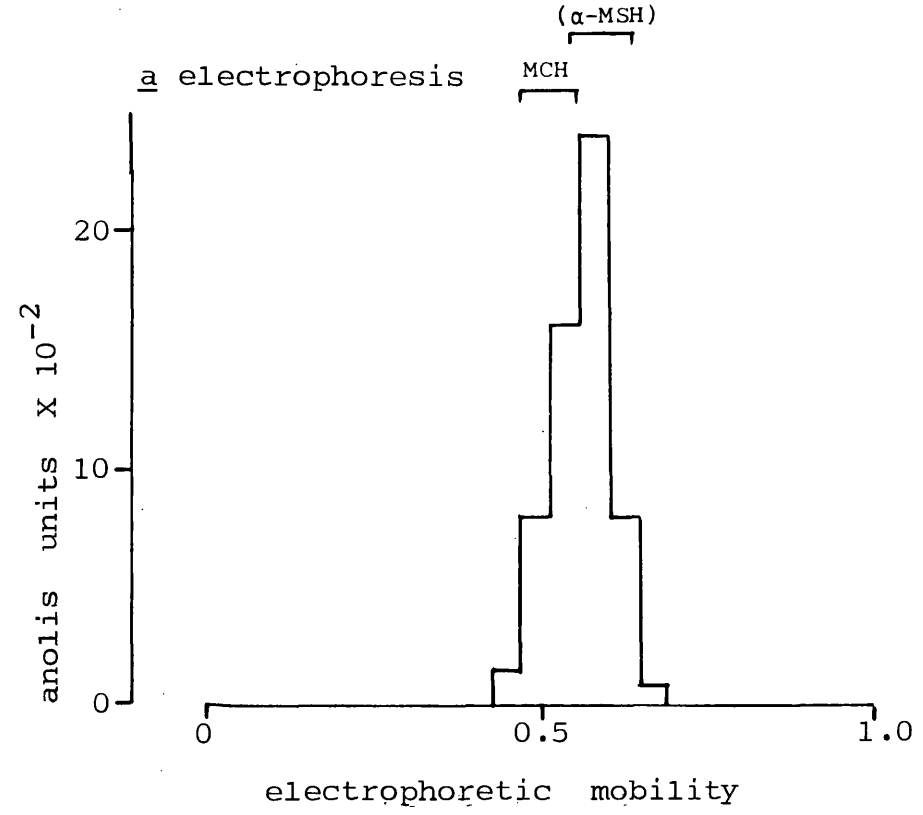


Fig. 6 An extract of ten trout pituitaries was prepared in electrophoresis buffer. After centrifugation, half the supernatant was applied as a 5cm band to each of two cellulose TLC plates. One was subjected to electrophoresis - the other to chromatography. The plates were scraped in 5mm bands and these fractions were eluted, lyophilized and bioassayed for MCH.

Fig.7 The ODS-Porasil extract described in Fig.5 was subjected to electrophoresis then chromatography on cellulose thin-layers. In each case bioassays were performed for MSH and MCH. In a separate track, α-MSH was run as reference standard - its position was visualized by spraying with fluorescamine. ^{125}I-α-MSH was present as internal standard and its recovery from these methods is given in Table 4.


(α-MSH)
MCH
a electrophoresis
anolis units X 10^{-2}
20
10
0
0
0.5
1.0
electrophoretic mobility

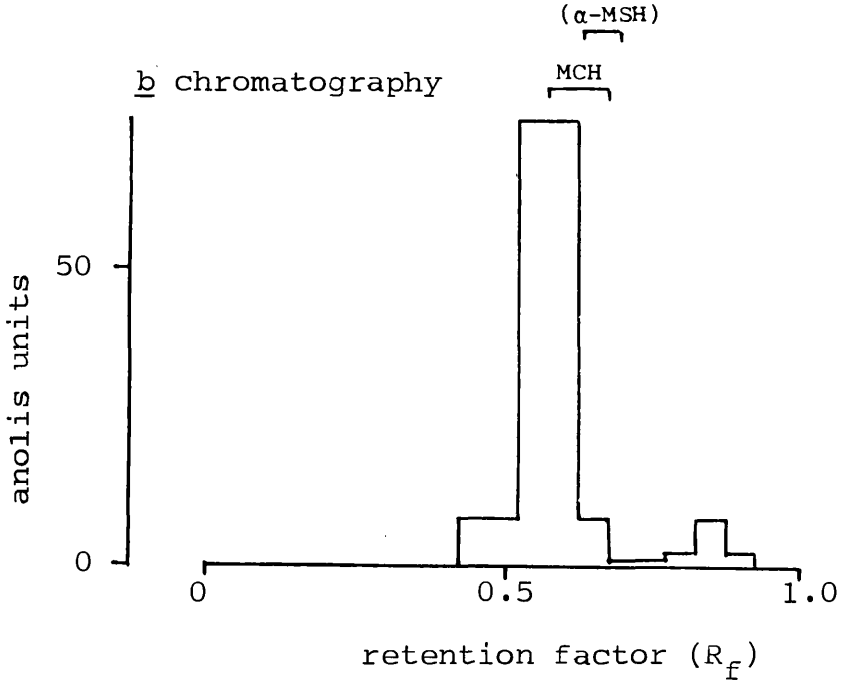

(α-MSH)
MCH
b chromatography
anolis units
50
0
0
0.5
1.0
retention factor (R_f)

Table 4

Recovery of ^{125}I α-MSH after ODS-extraction and electrophoresis / chromatography on thin-layers

stage of purification	% recovery	
	overall	of each step
ODS-Porasil extract (Fig. 5)	70	70
electrophoresis (Fig. 7a)	57	81
chromatography (Fig. 7b)	20	35

The recovery of ^{125}I-α-MSH was monitored throughout purification as a guide to the recovery of MCH and MSH.

e) Affinity chromatography with Concanavalin A

Concanavalin A (Con A) is a phytohaemagglutinin which will bind saccharides and polysaccharides bearing non-reducing terminal α-D-mannopyranoside or β-D-glucopyranoside residues. (For a review of the properties of this metalloprotein see Sharon and Lis (1972)). Glycoproteins or glycopeptides satisfying these criteria will also bind to Con A (e.g. pro-opiocortin,Krieger and Liotta, 1979). Were MCH to bind to Con A, this would prove a valuable tool in its purification. To determine this, an extract of 10mg of acetone-dried pituitary powder was prepared by sonication in 1.0ml of 0.1M ammonium acetate buffer pH 5.6 and applied to a 1.0ml column of Con A-Sepharose 4B. The column was then eluted at a flowrate of 3ml h^{-1} with 5ml each of: buffer, α-methyl-D-mannopyranoside 0.5M in buffer, and finally with 66% v/v acetic acid. The acetic-acid was used to elute non-specifically bound substances. One ml fractions were collected and lyophilised. For reconstitution, sugar-containing fractions were made-up in 2.5ml of distilled water and diluted with MCH-bioassay medium for assay. Other fractions were reconstituted directly in bioassay medium.

The only fractions to contain MCH activity were the first two of the initial buffer wash showing that MCH was not retarded or bound by Con A. In view of the

high specificity of Con A, failure of MCH to bind to it does not exclude the possibility that MCH is a glycopeptide.

f) Ion exchange chromatography

The cationic behaviour of MCH and MSH during electrophoresis suggested that cation-exchange chromatography might be used to seperate them. At low pH and low ionic strength (pH 4.8, 0.05M ammonium acetate) both bioactivities were bound by the cation exchanger SP-Sephadex C-25. In a pilot experiment a gradient from 0.05M ammonium acetate pH4.8 to 0.5M pH 7.0 was found to elute MCH and MSH bioactivity rather late in the gradient suggesting that there might be other peaks which remained irreversibly bound to the exchanger under these conditions. This was indeed found to be so since gradients to 1.0M pH 7.0 eluted further peaks of MSH bioactivity. The results of ion-exchange chromatography under these conditions are described fully in the next section.

3.3 Preparative ion exchange chromatography

Fig 8 shows the results of ion-exchange chromatography of crude acetic-acid extracts of both of the commercial salmon pituitary powders using gradients of ammonium acetate from 0.05M pH 4.8 to 1.0M pH 7.0. Good separation of MCH and MSH was obtained, and this method was also able to tolerate high sample loads (gram quantities). It was therefore used as the first chromatographic step for purification of MCH.

As in HPLC (Fig 4), ion-exchange separated MCH into two peaks (Fig 8); however, the elution positions of these peaks differed when extraction was performed at 100°C (b,c) instead of at room temperature (a). Subsequent analysis (section 3.6a) indicated the presence of N-terminally shortened forms of des-acetyl-α-MSH in the unheated extract (a). For this reason extraction with heat was adopted routinely to preclude proteolysis in the extracts. A further advantage of using heated extracts,evident from Fig 8 is the superior separation of MCH and MSH which was obtained.

In all cases, two peaks of α-MSH immunoreactivity were found. On the basis of their elution order, the first less basic peak was tentatively identified as α-MSH and the other as desacetyl-α-MSH.

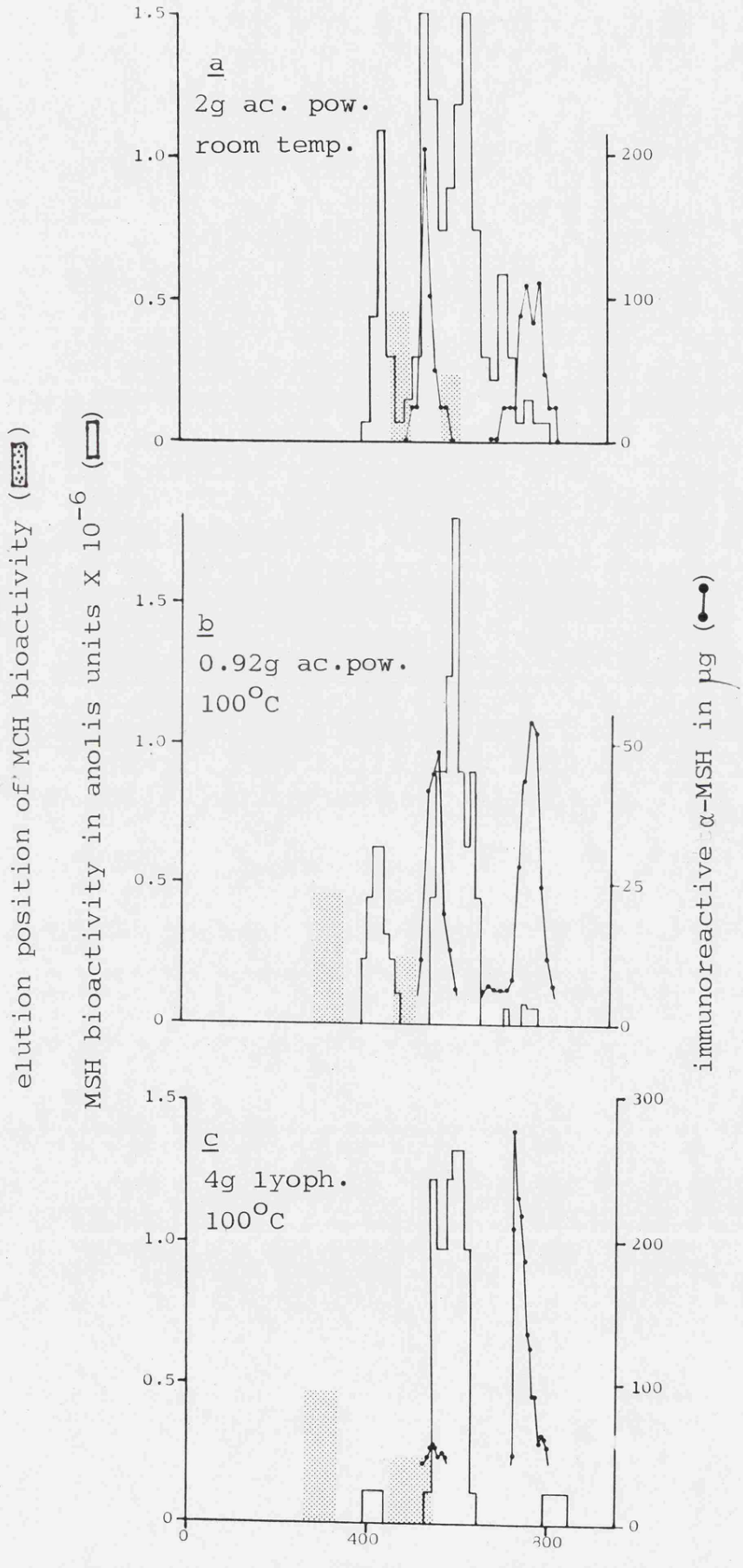
In the case of the heated extracts (b,c) "α-MSH" coincided with a shoulder of the major peak of MSH bioactivity indicating the presence of another MSH; possibly a type of β-MSH. This MSH comprised most of the Anolis melanophore-dispersing activity of the extracts.

In the lyophilised pituitaries (c) there was a marked preponderance of the desacetyl form of α-MSH. The significance of this is uncertain, but it is interesting that acetylation can enhance by 10-fold the biological potency of desacetyl-α-MSH in both the Anolis and frog bioassays (Ramachandran and Li (1967), frog; Mr. T. Bowley (personal communication), Anolis).

For further purification of MCH, the first MCH peaks of Fig 7a and c have been used; note that these appear to be different peptides. Peptides from Fig 7b were not subjected to further purification, this run was used only to check the reproducibility of the method.

Fig.8

Cation exchange chromatography of salmon MSH and MCH; the effect of heating the extracts . Extracts of the commercial pituitary powders in 1M acetic acid were lyophilized and reconstituted in 70ml of starting buffer using a homogeniser. The resulting suspension was centrifuged at 90,000g for 30min and the supernatant was applied to a 30 x 2.5 cm column of SP-Sephadex C-25 equilibrated in the same buffer and run at a flowrate of 40ml h^{-1}. After application of the sample, the column was washed with a further 200ml of starting buffer to elute non-absorbed solutes. This was followed by an 800ml linear concentration-gradient of ammonium acetate (from 0.05M pH 4.8 to 1.0M pH 7.0). No MSH or MCH bioactivity could be detected in the initial 200ml wash, and only the fractions collected after application of the gradient are shown. In peptides purified from a, there was evidence that proteolysis had occurred. In an effort to avoid this, extraction was subsequently performed in boiling acetic acid (in b and c). Stippled histogram = MCH . The MCH bioassay was performed semi-quantitatively - enough to identify which peak was the major.


a
2g ac. pow.
room temp.
b
0.92g ac.pow.
100°C
c
4g lyoph.
100°C
1.5
1.0
0.5
0
200
100
50
25
300
400
elution position of MCH bioactivity
MSH bioactivity in anolis units X 10⁻⁶
immunoreactive α-MSH in µg
elution volume in ml

3.4 Partial purification of MCH

a) Purification of MCH from acetone-dried pituitaries

This purification is summarized in the flow diagram (Fig 9).

Two peaks of MCH bioactivity were recovered from ion-exchange chromatography of a crude acetic-acid extract of acetone-dried pituitaries (Fig 8a). The first peak was taken for further purification because it contained most of the MCH bioactivity and was relatively free of MSH bioactivity and α-MSH immunoreactivity. The fractions comprising this peak were pooled, lyophilised and reconstituted in 1M acetic acid. The acetic acid solution was then subjected to gel-filtration on a column of Sephadex-G-25 eluted in the same solvent (Fig 10). A single peak of MCH bioactivity was recovered which did not correspond to any peak in the absorbance trace. Its elution position suggested that it was intermediate in size between the synthetic peptides : desacetyl-α-MSH (1623 daltons) and hACTH 1-24 (2934 daltons). This is consistent with earlier findings from chromatography of crude extracts on Bio-Gel P2 (section 3.2a) and with subsequent estimates from Sephadex G-25 (section 3.10) which suggest a molecular weight of 1700 daltons.

The MCH bioactive fractions from gel-filtration (Fig 10) were pooled in a siliconized borosilicate-

glass vial and stored frozen at -20°C. This solution was applied directly to a semi-preparative scale HPLC column (HPLC system 1) which was eluted with a gradient of methanol in 1% TFA (Fig 11). Since peptides from gel-filtration in 1M acetic-acid were found to concentrate on the HPLC column, this obviated the need for concentration by lyophilisation. Both MCH and MSH bioassays were performed on the resulting fractions. It was evident from the absorbance trace (Fig 11) that useful purification from non-bioactive peptides had been obtained, however, this method was only partly effective at purifying MCH from residual contaminating bioactive MSH (for a quantitative comparison see Table 5).

The MCH-containing fractions from this HPLC run were pooled, and to allow application to HPLC system 2, the methanol concentration was reduced by evaporation under reduced pressure until the sample was 1/5 its original volume. In later purifications it was found to be sufficient to reduce the concentration of organic modifier (acetonitrile or methanol) by dilution with an equal volume of deionised water.

Aliquots of the concentrated sample were then applied to HPLC system 2 - an analytical-scale column of Nucleosil-5-ODS eluted with gradients of acetonitrile in acid phosphate buffer. First, to establish what sort of gradient would best resolve all peptide

components of the sample, small aliquots were sacrificed in analytical runs - modifying the gradient each time to optimise separation (not shown).

Under the conditions thus established, 1/10 of the sample was then chromatographed and bioassays were performed for MCH and MSH (Fig 12a). MCH was found to co-elute with a peak of MSH bioactivity. Even at this stage of purification, MCH still did not correspond to an absorbance peak. Previously, at 280nm, this could have been due to absence of tryptophan in the peptide, but all peptides absorb at 225nm - the wavelength used here.

A further aliquot of 1/10 of the sample was then chromatographed with a slightly modified gradient to try to improve resolution in the region where MCH eluted (Fig 12b). This achieved some separation from contaminating MSH bioactivity but was unsuccessful at isolating MCH.

The remainder (65%) of the sample (after ion-exchange, gel-filtration and HPLC system 1) was committed to chromatography under the conditions of Fig 12b and the MCH-containing fractions were collected, diluted 1:1 with water and reapplied to the same column. This served to reduce the peptide content of the sample optimising sample-load for re-chromatography.

For re-chromatography on HPLC system 2, rather than eluting with a gradient, the column was eluted with a constant concentration of 17% acetonitrile 0.06M phosphate buffer pH 2.1 (isocratic elution). Bioassay showed that MCH eluted after 39min under these conditions but the resulting peaks were so dilute as to be undetectable by their absorbance. Since the absorbance trace could not be used to assess purity, N-terminal determinations and amino-acid analyses were performed on this MCH preparation.

Before such analyses could be performed it was necessary first to desalt the peptide preparation. This was achieved by ODS-extraction (see methods), salt-free peptides were recovered from the resulting solution in 80% methanol 1% TFA by evaporation to dryness under reduced pressure.

N-terminal determinations by the dansyl method showed the MCH preparation to be heterogeneous : N-terminal Asp and Lys were found together with internal Lys and Tyr. Amino-acid analyses by the dual-isotope method confirmed the presence of these residues and that the preparation was heterogeneous since non-integral ratios were found. Because the preparation was impure, none of these findings necessarily pertain to MCH wich could still be a minor component of the preparation.

Solvent blanks prepared in parallel with the MCH sample by drying samples of 80% methanol 1% TFA were found to contain undetectable levels of amino-acids in both the N-terminal and the amino-acid analyses.

No further purification was obtained by re-chromatography on HPLC system 2 under isocratic elution as judged by the specific activity (i.e. bioactivity per unit mass) of the peptide preparation obtained.

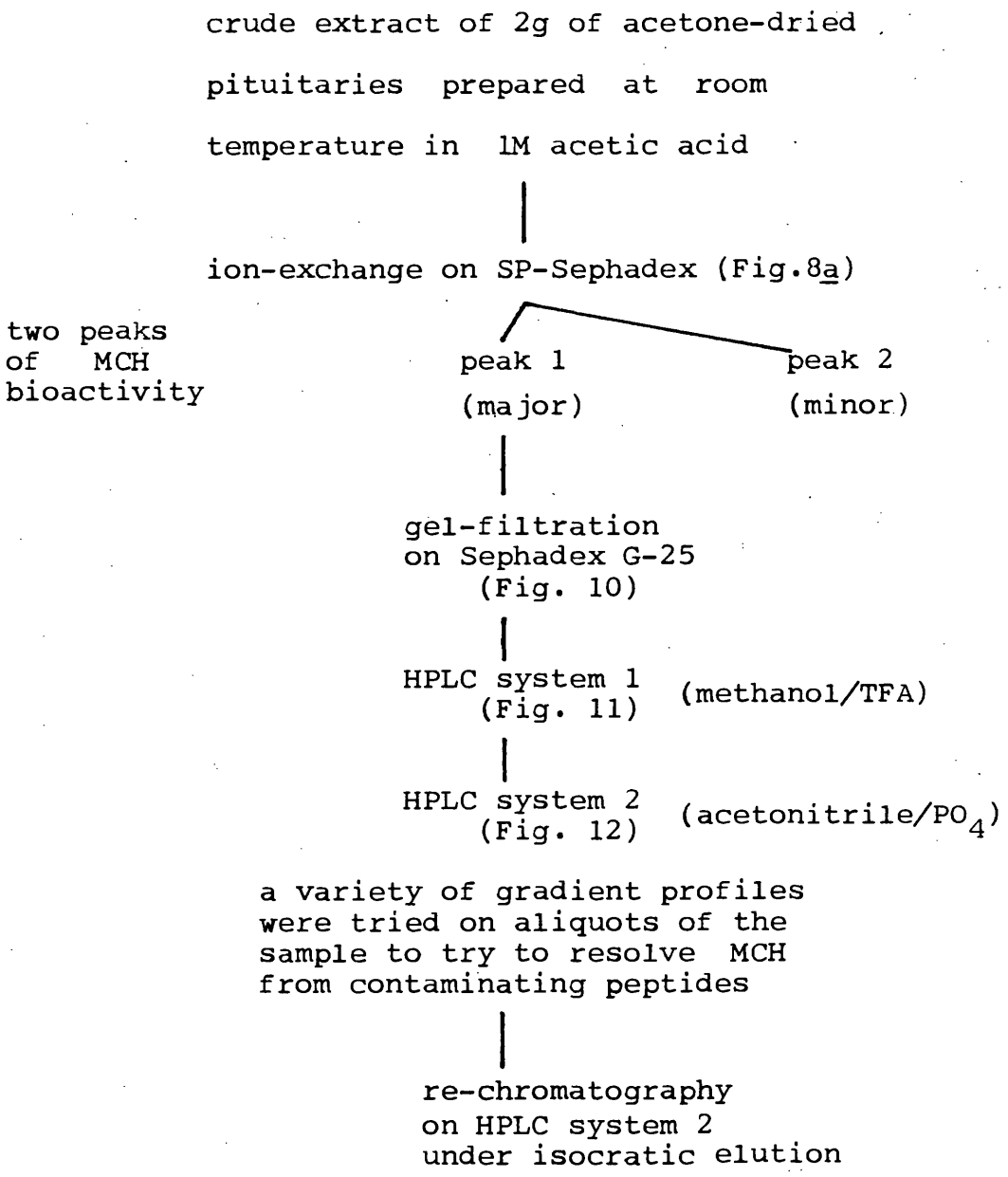
Overall purification and yield

In the purest preparation of MCH obtained (i.e that from Fig 12b), 25% of the original biological activity was recovered with 88µg of peptide. Since 2g of acetone-dried pituitaries (equivalent to about 10g of fresh pituitaries - see section 2.3) were used for this purification, this represents an overall increase in specific activity of about 28 000-fold relative to that in the fresh tissue. i.e. The purification factor was about 28 000.

Separation of MCH from MSH

Table 5 shows the degree of contamination of MCH with bioactive MSH throughout purification. Very small but significant amounts of MSH bioactivity persisted even in the most pure preparations of MCH obtained. This residual contaminant is not α-MSH since calibrations

Fig. 9 Flow diagram for the purification of MCH from acetone-dried pituitaries



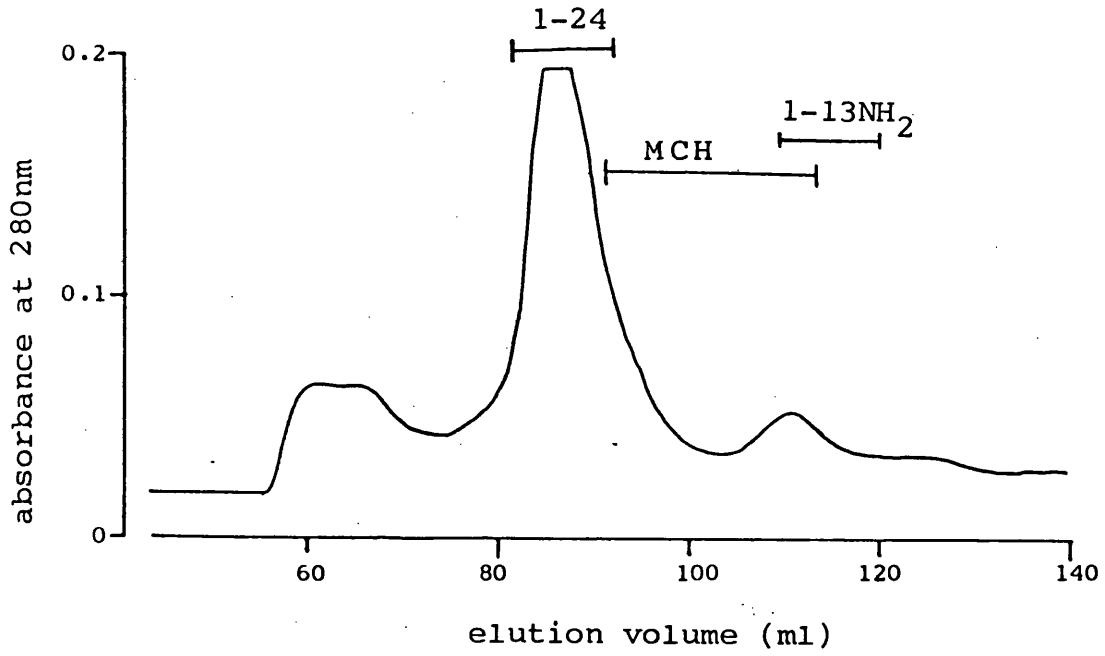


Fig. 10

Gel-filtration of MCH from ion-exchange of 2g of acetone-dried pituitaries (Fig. 8) on Sephadex G-25. The lyophilized sample was reconstituted in 3ml of 1 M acetic-acid and applied to the 100 X 1.6cm column which was eluted with the same solvent at a flowrate of 7.7 ml h^{-1}. Fraction volume was 2ml. The elution positions of the synthetic peptides desacetyl-α-MSH (i.e. 1-13NH_2) and ACTH 1-24 were determined in a separate run on the same column.

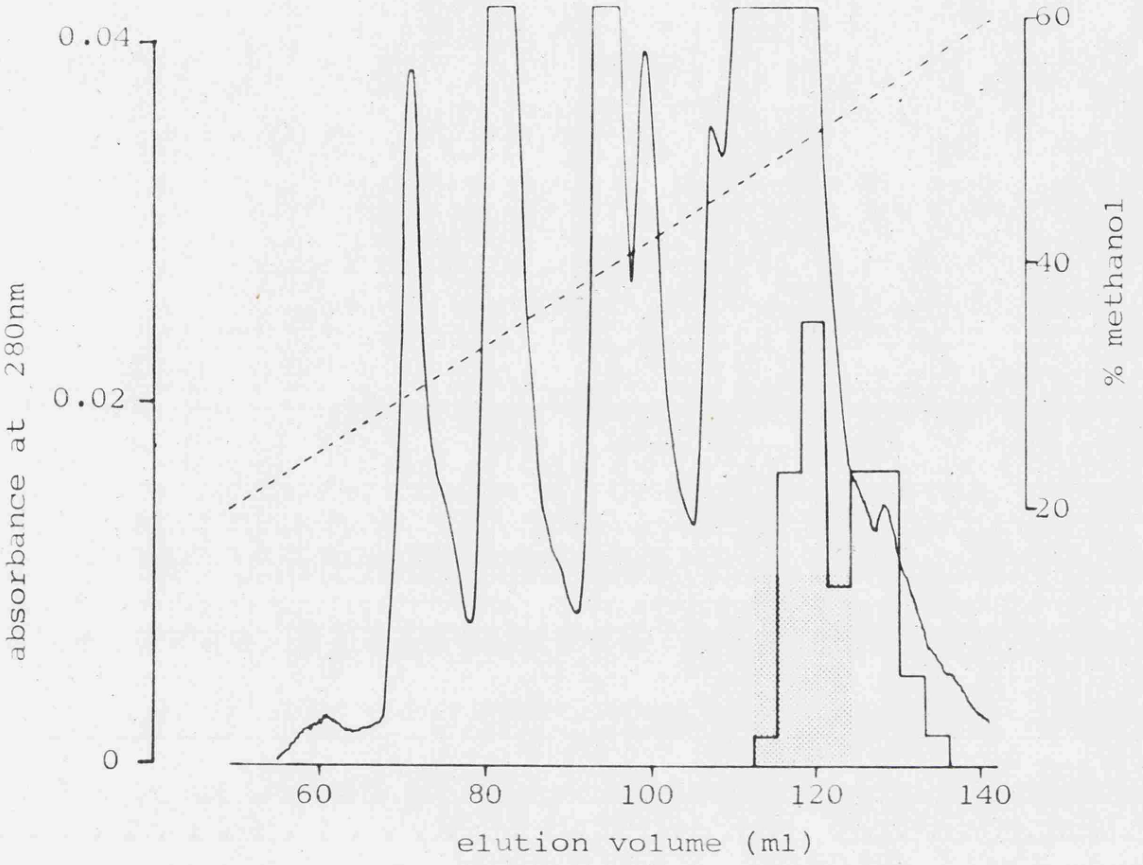


Fig. 11 Semi-preparative scale HPLC (system 1) of MCH from 2g of acetone-dried pituitaries after ion-exchange and gel-filtration. Open histogram = bioactive MSH, stippled histogram = MCH. The sample from gel-filtration (in 1M acetic acid) was applied directly to a 300 X 8mm column of Partisil-10-ODS operated at a flowrate of 3ml min^{-1}. The sample was followed by a simple linear gradient (broken line) from 0-80% methanol. Peptides were detected by their absorbance at 280nm.

Fig. 12 HPLC (system 2) of MCH from 2g of acetone-dried pituitaries after purification by ion-exchange, gel-filtration and HPLC (system 1). Open histogram = bioactive MSH, stippled histogram = MCH. Aliquots of one tenth of the sample were applied to a 100 X 5mm column of Nucleosil-5-ODS operated at a flowrate of 1.0 ml min^{-1} with sucessive modification of the gradient profile : a and b. Detection wavelength was 225nm, 0.32 absorbance units = full scale. Fractions of 0.2 ml were collected.

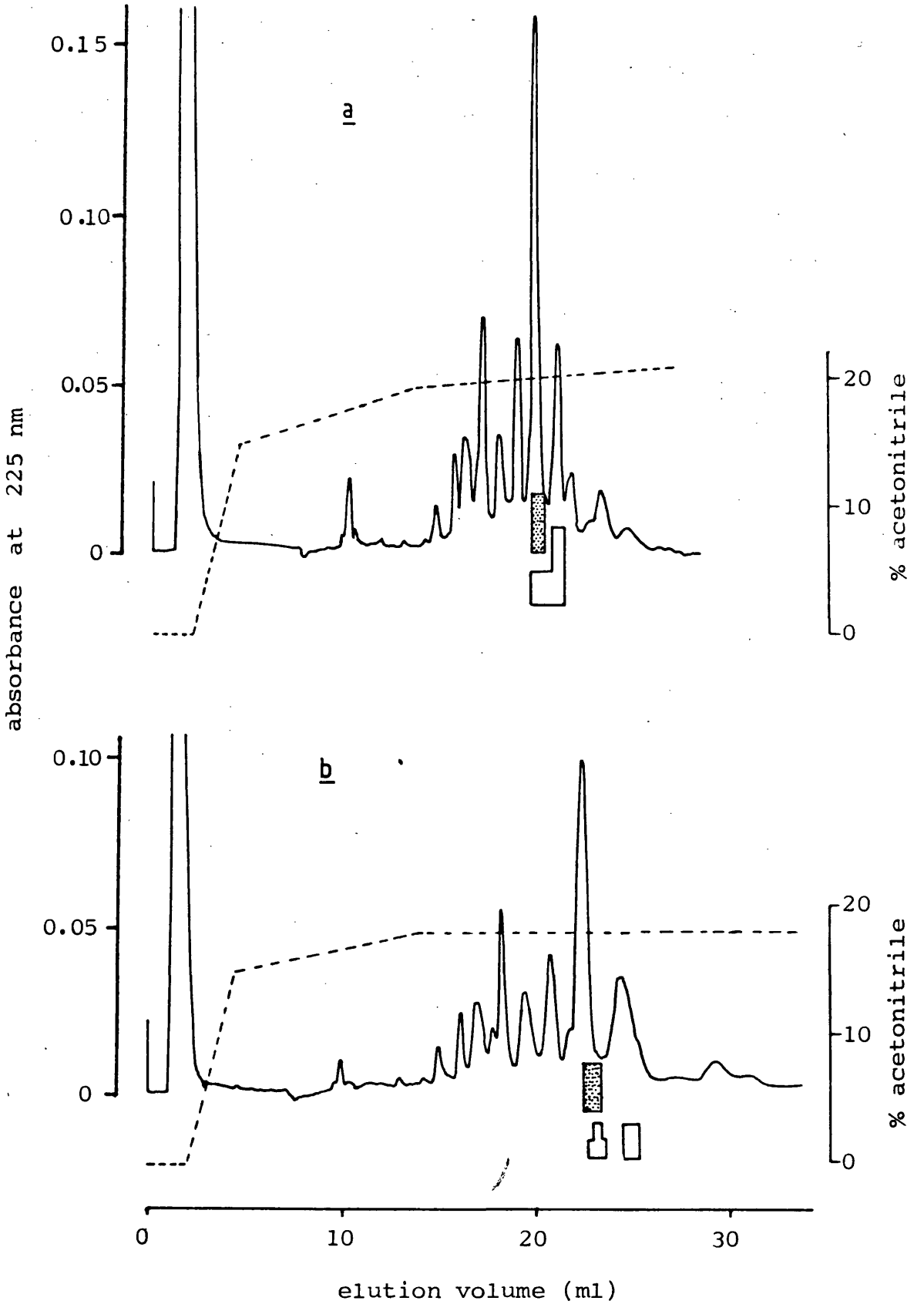

a
b
0.15
0.10
0.05
0
0.10
0.05
0
absorbance at 225 nm
20
10
0
% acetonitrile
20
10
0
% acetonitrile
0
10
20
30
elution volume (ml)

Table 5

Purification of MCH from 2g of acetone-dried pituitaries: yield and contamination with bioactive MSH.

stage of purification	yield of MCH*	yield of MSH**	
	%	%	µg
crude extract	100	100	2 400
ion-exchange and gel-filtration	60	0.33	8.0
HPLC (system 1)	35	0.40	9.5
HPLC (system 2) (gradient runs)	25	0.014	0.34
HPLC (system 2) (isocratic run)	9	0.0063	0.15

*= determined by comparison to the bioassay standard described in "methods" (section 2.5).

**= determined by comparison to α-MSH standard.

Recovery of MCH was estimated quantitatively after pooling fractions on the basis of qualitative MCH bioassay. This was necessary to reduce the number of samples to be assayed to within the capacity of the bioassay (see section 3.5a).

of the HPLC system 2 with synthetic α-MSH had shown this to elute later than MCH and its contaminating MSH bioactivity.

b) Purification of MCH from lyophilised pituitaries

The procedure used for purification of MCH from the heated extract of lyophilised pituitaries was essentially the same as that for purification from the acetone powder. Since quantitative use of the MCH bioassay had shown that MCH could be recovered from all of the methods used, in this purification bioassays were used qualitatively - sufficient to locate peaks of MCH among the chromatographic fractions. In this purification mercaptoethanol was included at 0.1% v/v for extraction and 0.01% for chromatography to avoid methionine-sulphoxide formation (see section 3.6a).

Ion-exchange chromatography of the crude extract is described in section 3.3 Fig 8c. Two MCH peaks were found as before but they eluted earlier indicating that they were less basic than those from the unheated extract of acetone-powder. The first of these was taken for further purification since it was essentially free of MSH.

Gel-filtration of this sample on Sephadex G-25 gave a single peak of bioactivity which eluted in the same

position as that purified from the acetone powder (see previous section). Thus, although this MCH appeared less basic than that purified from the acetone powder, it had the same apparent molecular weight. As before, the MCH bioactivity did not correspond to an absorbance peak.

When the MCH-containing fractions from gel-filtration were applied, without lyophilisation direct to HPLC system 2 (Fig 13) the sample gave a complex absorbance profile. Since the initial extraction had been performed at 100°C, it is unlikely that the complexity of this mixture was due to *in vitro* proteolysis, a contingency which could not be excluded before. However, when these HPLC fractions were bioassayed for MCH, no activity was detected. The reason for this loss of bioactivity is unknown. Experiments on the stability of MCH in crude extracts (section 3.1) had indicated that it was not easily inactivated chemically.

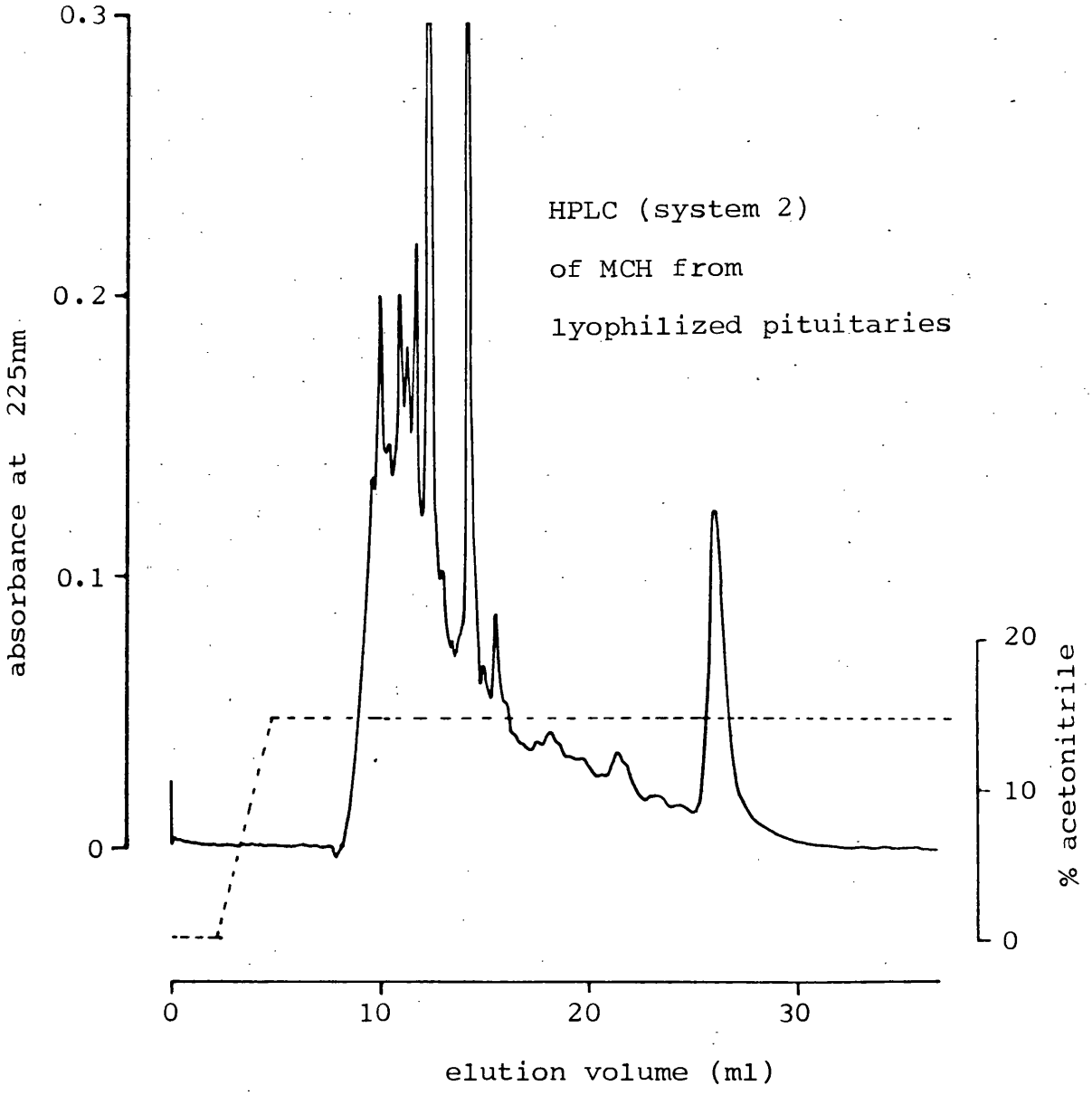


Fig. 13 When MCH from 4g of lyophilized pituitaries was run on HPLC (system 2) as in Fig. 12 , peptides eluted earlier than expected resulting in poor resolution. The peptide-containing fractions were therefore diluted with an equal volume of water and re-run as shown with a modified gradient. No MCH was detected in fractions from this run or in the side-fractions from the initial run. In each case the gradient was to 60% acetonitrile although only the region where peptides were detected is shown.

3.5 The measurement of melanophore-concentrating activity

a) The MCH bioassay

The MCH bioassay has proved difficult to use quantitatively because of unreliability in the quality of scales, specifically;

1) The melanophores of trout-scales *in vitro*, though dispersed initially, undergo slow spontaneous concentration to a variable extent in the absence of any applied stimulus.

2) There is great variation between scales of the same fish in the response of their melanophores to a given concentration of MCH.

The spontaneous concentration which occurrs *in vitro* was minimised by the routine inclusion of 10^{-4}M Rogitine (an α-adrenergic antagonist) in the bioassay medium - which suggests it was caused by endogenous catecholamines. Spontaneous concentration does not occurr uniformly to all melanophores but appears to affect particular scales at random. Thus, to be sure that an observed response is due to an applied stimulus (e.g. added MCH) several scales have to be tested. This is so even for a qualitative determination i.e. to decide whether or not MCH is present. The bioassay used quantitatively consists of qualitative determinations at a variety of dilutions to establish the "threshold dilution" (section2.5); as this

condition is approached, more scales are required for each successive test. In my experience this limits quantitative use of the bioassay to a few samples (up to six).

Since MCH-activity is measured in trout-units and since this measurement is derived from a dilution factor (section 2.5) it is a discrete or quantized variable; this precludes the use of standard-error estimates (i.e. mean ± SEM) in describing the precision of the data, since this is only valid for continuous variables.

Inter-assay variation

A study of inter-assay variation using the standard preparation described in section 2.5 showed that in nine separate assays the apparent MCH-content of 1mg of acetone-dried pituitary powder ranged from 20 - 300 trout-units with a mean of 93 trout-units. i.e. On average, a solution of 1mg of the pituitary powder in about 9.3ml was just detectable.

b) The measurement of melanophore responses by microdensitometry

Two major disadvantages of the MCH bioassay are: the quantized nature of the measurement obtained, and the inacurracy of the subjective assessment of melanophore

responses. Microdensitometry was used in an effort to improve these aspects of the bioassay by generating continuous data and by measuring melanophore responses accurately and objectively. In the ensuing experiments, for convenience, noradrenaline has been used to induce melanophore-concentration.

Fig 14 shows the precision with which area-measurements can be made on a single living melanophore using the microdensitometer, and also demonstrates that the response is not the "all or none" type since a stable state of intermediate concentration was attained. Unfortunately, the recovery-time of trout-scale melanophores *in vitro* (>30 min) is too long to allow replicate observations to be made on the same melanophore. Therefore, to investigate the dose-response relationship to noradrenaline, the same approach was used as for the MCH bioassay: scales bearing dispersed melanophores were incubated in test-solutions, and after a prescribed time (in this case 1h) the area of the melanophores was measured.

A dose-response curve was constructed by plotting change in melanophore area (% response) as a function of noradrenaline concentration (Fig 15). Extensive spontaneous melanophore-concentration occurred in the absence of Rogitine which obscured the effect of added noradrenaline. However, in this case, the presence of 10^{-4}M Rogitine prevented spontaneous

concentration. Under these conditions, added noradrenaline caused melanophore-concentration in a dose-dependant manner and at 10^{-5}M - completely overcame the effect of 10^{-4}M Rogitine. The observation that noradrenaline was able to overcome the blocking effect of Rogitine indicates that Rogitine acts as a competetive antagonist of noradrenaline as it does on the tailfin melanophores of Pterophyllum eimekei (Finnin et al 1979).

The apparent log-linear nature of the lower part of the dose-response curve indicates that the area measurement might be a good basis for a quantitative bioassay. However, there was great variation between trout-scale melanophores in their response to a given concentration of noradrenaline and large numbers of observations were necessary for each data-point (n=25).

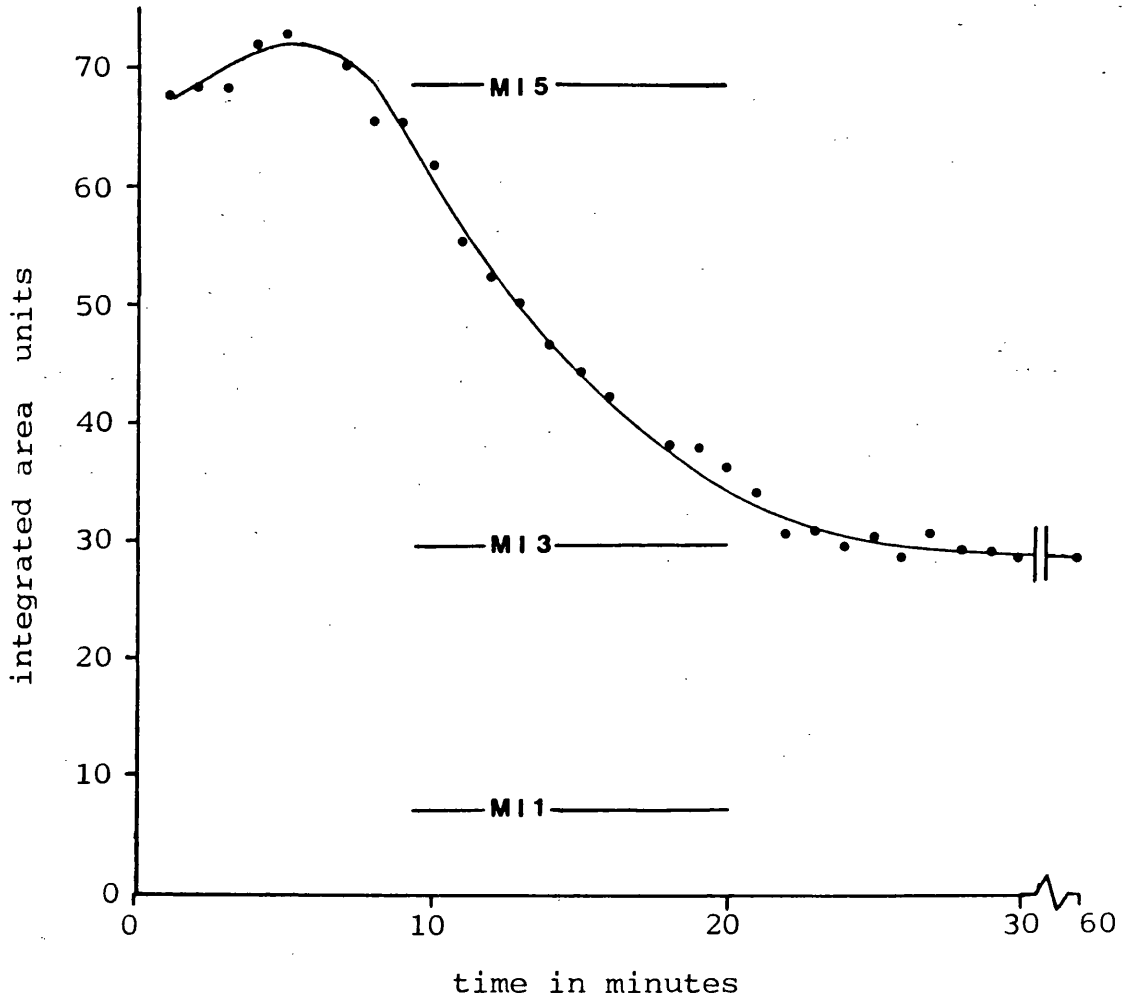


Fig. 14 The response of a single trout-scale melanophore to 10^{-6}M noradrenaline measured with the flying-spot microdensitometer. MI = melanophore index.

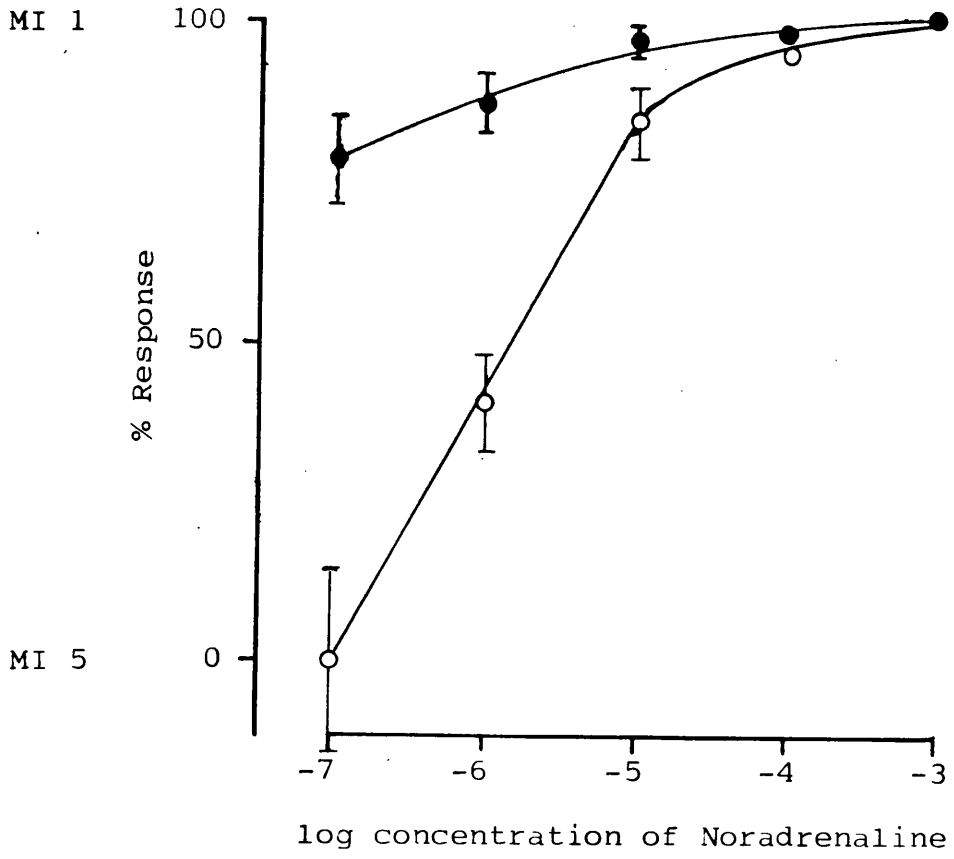


Fig. 15 The effect of noradrenaline on trout-scale melanophores in the presence (O) and absence (●) of 10^{-4}M Rogitine. Five melanophores selected at random from each of five scales were measured for each data-point; values are means ± SEM, n=25. MI=melanophore-index.

3.6 Purification of MSH

Introduction

Since both MCH and MSH are implicated in the control of teleost melanophores, it is important to our understanding of how their actions are co-ordinated to have some idea of the amounts of each which exist in the pituitary in terms of both mass and biological-activity.

The availability of a radioimmunoassay for α-MSH has enabled measurement of the mass of "immunoreactive α-MSH" in crude pituitary extracts (section 3.11). However, when the identity of an immunoreactive substance is uncertain and therefore its degree of cross reactivity is unknown, large errors can result in its estimation by radioimmunoassay, and ultimately its precise identity can be established only by isolation and chemical analysis.

From the results of section 3.2 on the chromatographic behaviour of MCH and MSH, it was evident that MCH was a peptide with physicochemical properties similar to those of the MSH's. It was considered therefore that purification of the MSH's of trout pituitary in parallel with MCH would provide a useful comparison since susceptibility to loss or damage during purification should be similar.

Thus, to enable comparison to MCH in terms of mass per pituitary and to establish the identity of the immunoreactive MSH's - one of these was purified. The second immunoreactive-α-MSH from ion-exchange (Fig 8) was taken because at this stage it was purer than the first as judged by the absorbance trace (not shown).

a) Purification of desacetyl-α-MSH like peptides from acetone-dried pituitaries

The purification of these peptides is summarised in the flow diagram (Fig 16).

The second immunoreactive α-MSH peak from ion-exchange chromatography (Fig 8a) was further purified by gel-filtration on Sephadex G-25 (Fig 17). MSH bioactivity was confined to the main absorbance peak of the chromatogram. The specific-activity of this peptide preparation (i.e. bioactivity per unit mass) was 10% that of synthetic α-MSH measured in the same bioassay, i.e. the same as that of desacetyl-α-MSH (see section 3.3). However, its elution position suggested it was somewhat smaller than desacetyl-α-MSH.

N-terminal determination by the dansyl method showed this peptide to be heterogeneous, so an aliquot (1/10) was subjected to further purification on HPLC system 2 (Fig 18). The absorbance trace showed only two major

peptide components - A and B. No bioassay was performed since it was evident from Fig 17 that the biologically active peptides had been major components of the applied mixture.

Structural analysis

The results of amino-acid analyses, N-terminal determinations and α-MSH radioimmunoassay of peaks A and B are given in Table 6. Their strong immuno-reactivity with the C-terminally directed α-MSH antiserum suggested similarity of their C-termini with α-MSH / desacetyl-α-MSH i.e. -Lys-Pro-Val-$CONH_2$. The amino-acid compositions of peptides A and B were similar to that which would be expected for α-MSH or desacetyl-α-MSH. Indeed, that of B was essentially identical.

Since B had a free N-terminal serine residue, this would suggest it was desacetyl-α-MSH since the acetyl group at the N-terminal of α-MSH renders it unreactive to dansyl chloride. However, desacetyl-α-MSH is a C-terminal amide and with the data considered so far, peptide B could be either a C-terminal acid or a C-terminal amide (since the acid hydrolysis of peptides converts amides to acids).

From their amino-acid compositions (Table 6) peptide preparation A would appear to be a mixture of N-

terminally shortened derivatives of B i.e.;

B

$$\overset{1}{NH_2}\text{-Ser-Tyr-Ser-Met-Glu-His-Phe-}\overset{5}{}\qquad\qquad\overset{10}{}\qquad\qquad\overset{13}{}$$
$$\text{-Arg-Trp-Gly-Lys-Pro-Val-COOH/CONH}_2$$

A (i) major

$$\overset{4}{NH_2}\text{-Met-Glu-.....}\overset{13}{}\text{Val-COOH/CONH}_2$$

(ii) minor

$$\overset{2}{NH_2}\text{-Tyr-Ser-Met-Glu-.....}\overset{13}{}\text{Val-COOH/CONH}_2$$

Evidence from mass-spectrometry (see section 3.7a) suggested that peptide A(i) was a C-terminal amide. Since peptides A(i),(ii) and B co-eluted during purification on ion-exchange chromatography (Fig 8a) it is likely that they all were C-terminal amides.

The N-terminal Met residue of A(i) appeared from mass-spectrometry to be Met sulphoxide. Oxidation to the sulphoxide could easily have occurred during purification and / or storage. The sulphoxide was not detected by amino-acid analysis because of the presence of mercaptoethanol in the hydrolysing acid (6M HCl) which reduces this back to methionine.

To avoid oxidation of methionine in later purifications, mercaptoethanol was included at 0.1% v/v for extraction and 0.01% v/v for chromatography. To detect Met sulphoxide should it occurr, hydrolysis of peptides

Fig. 16. Flow diagram for the purification of α-MSH-like peptides from acetone-dried pituitaries

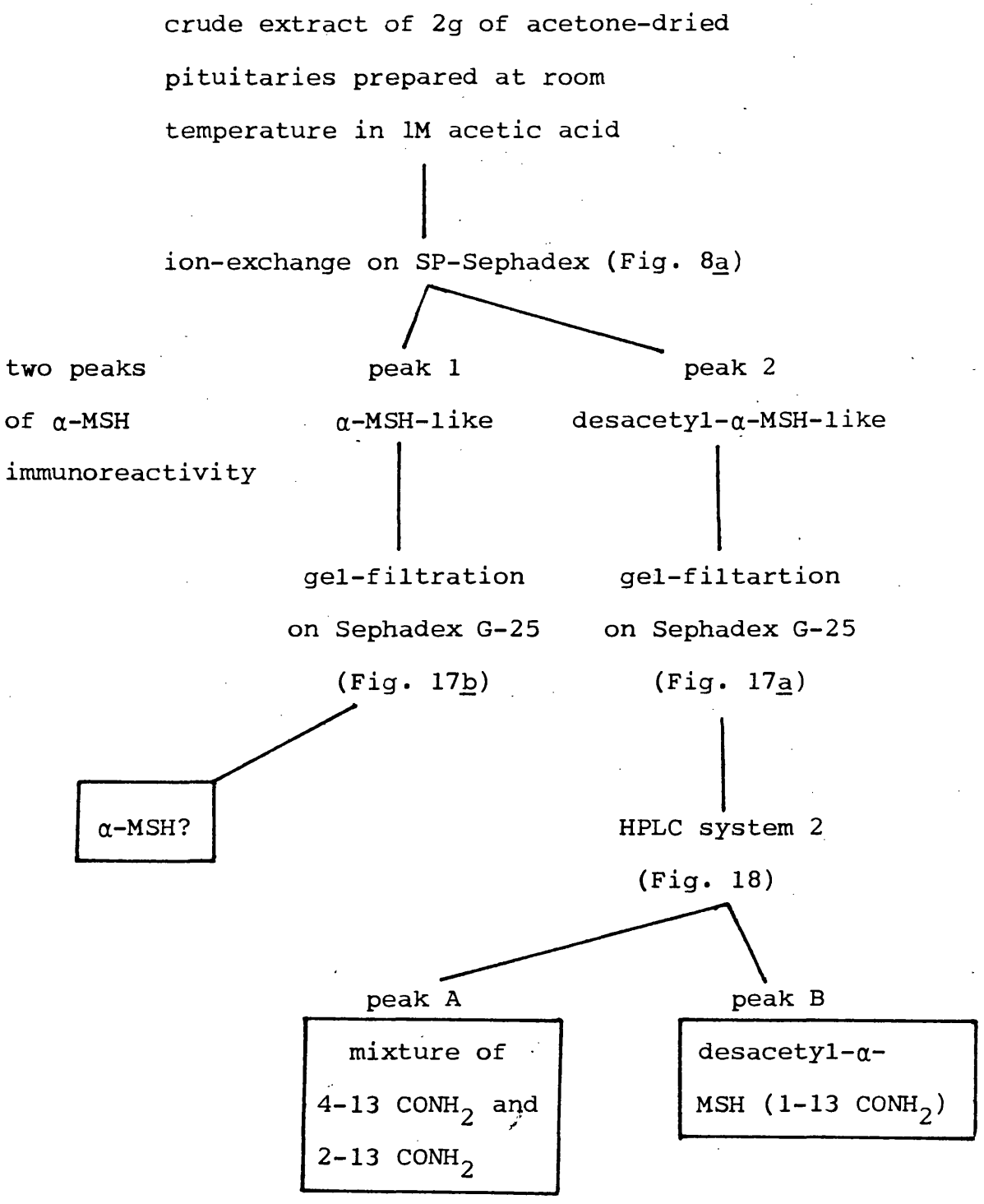
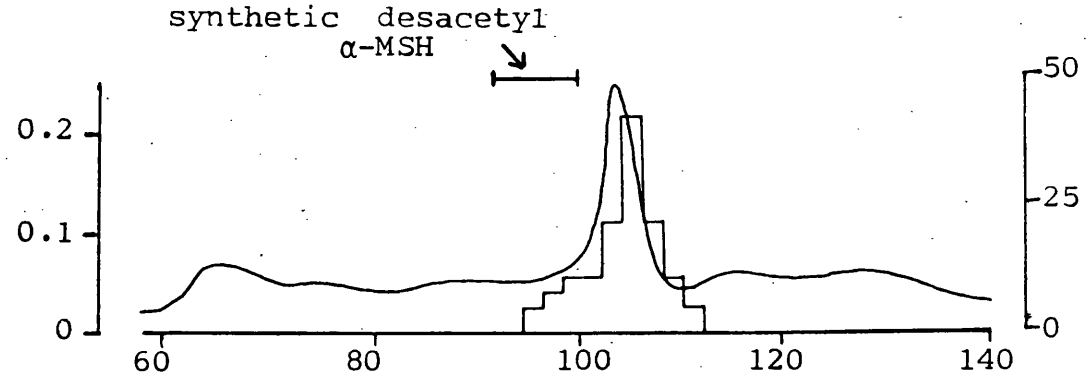


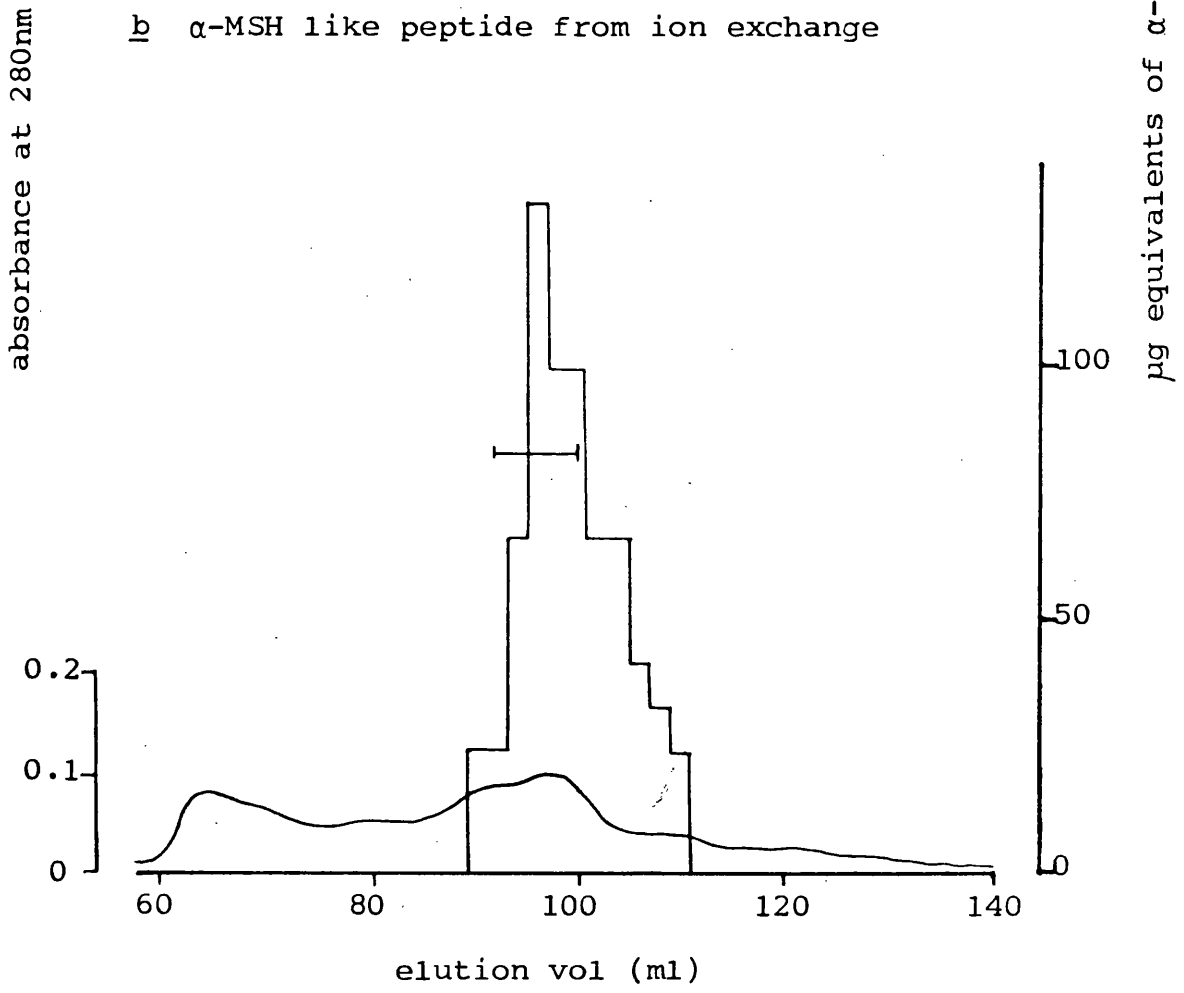
Fig. 17

Sephadex G-25 gel-filtration of immunoreactive -α-MSH's from ion-exchange of 2g of acetone-dried pituitaries (Fig 8a). The lyophilised samples were reconstituted in 3ml of 1M acetic acid and applied to the 100 X 1.6cm column which was eluted with the same solvent at a flowrate of 7.2ml h^{-1}. 2ml fractions were collected and assayed for MSH using the Anolis bioassay. In a, the desacetyl-α-MSH-like peptide from ion-exchange appeared smaller (i.e. eluted later) than did synthetic desacetyl-α-MSH run on the same column. Therefore, since its identity was still uncertain, it was committed to further purification on HPLC (Fig 18). In b the α-MSH-like peptide was similar in size to synthetic desacetyl-α-MSH (and therefore similar in size to α-MSH). The absorbance trace suggested that this peptide was still impure, nevertheless it was more biologically potent than the desacetyl-α-MSH-like peptide. These observations further support its putative identity as α-MSH.

a desacetyl-α-MSH like peptide from ion-exchange



b α-MSH like peptide from ion exchange



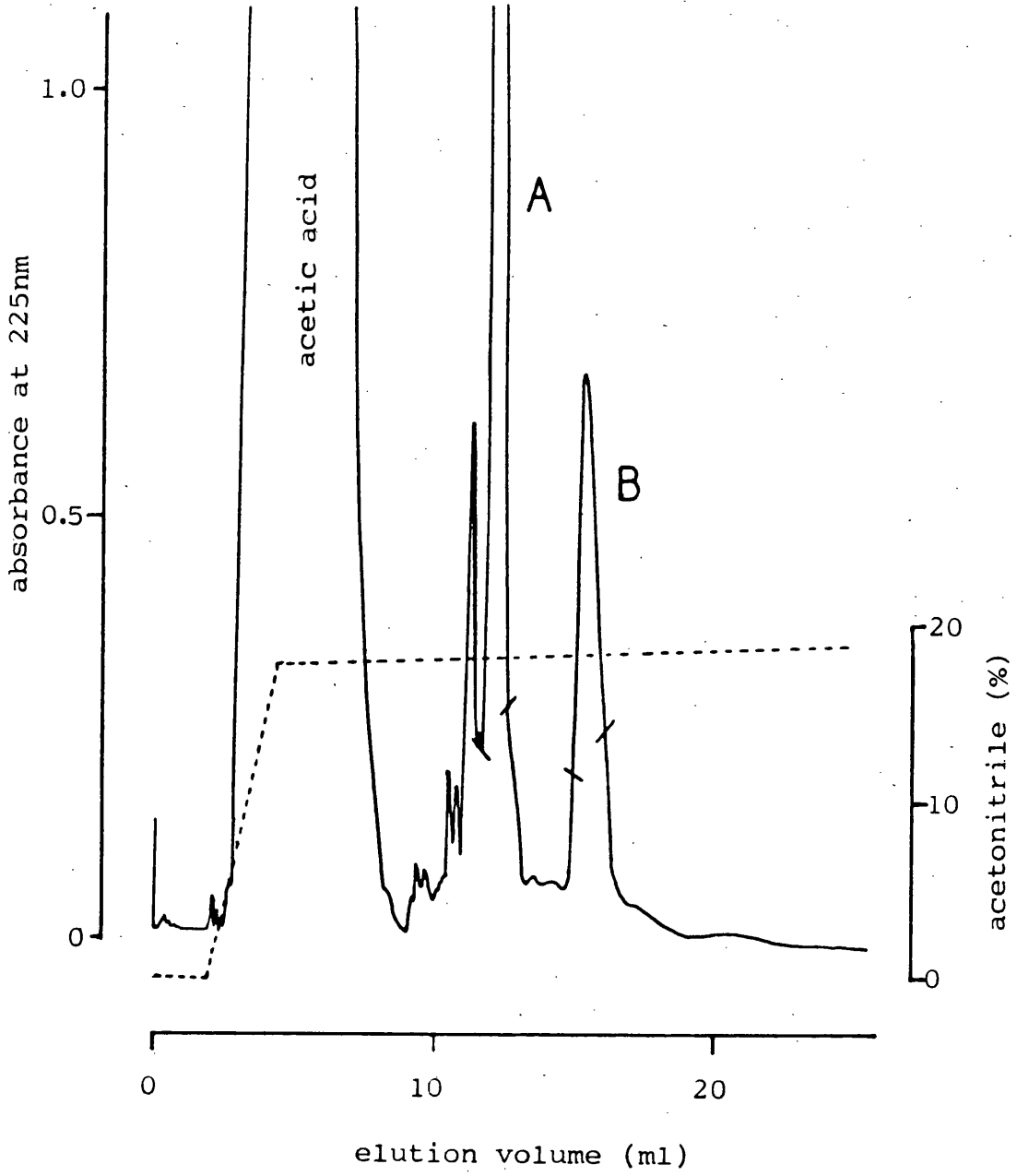


Fig. 18 HPLC (system 2) of desacetyl-α-MSH-like peptide obtained from ion-exchange and gel-filtration of an unheated extract of 2g of acetone-dried pituitaries. One tenth of the sample from gel-filtration (in 1M acetic acid) was applied directly to a 100 X 5mm column of Nucleosil-5-ODS operated at a flowrate of 1 ml min^{-1}.

Table 6 Amino-acid composition, yield, and immunoreativity of desacetyl-α-MSH-like peptides purified from 2g of acetone-dried pituitaries.

	peptides from Fig. 18		result which would be expected for α-MSH or desacetyl-α-MSH	
	peptide A	peptide B		
Ser	0.4	1.6	2	
Tyr	0.5	0.9	1	
Met	0.8	0.9	1	
Glx	1.1	1.0	1	
His	1.0	0.9	1	
Phe	1.0	0.9	1	
Arg	1.0	0.9	1	
Trp	present	present	1	
Gly	1.0	1.0	1	
Lys	1.0	1.0	1	
Pro	1.0	1.1	1	
Val	1.0	0.9	1	
N-terminal	none detected but internal Lys present	N-terminal Ser internal Lys and Tyr	none, but internal Lys and Tyr	N-terminal Ser plus internal Lys and Tyr
Amount ofpeptide recovered	227nmol (about 380µg)	147nmol (239µg)	-	
*%crossreactivity relative to α-MSH in α-MSH RIA (approximate)	99%	45%	100%	70%

*% crossreactivity was calculated by expressing the amount of peptide recovered determined by RIA as a percentage of that detected by amino-acid analysis.

Acid hydrolysis for amino-acid analyses was performed in 6M HCl + mercaptoethanol. Analyses were performed in duplicate by the dual isotope method (section 2.16)on samples of hydrolysate corresponding to about 300pmol of peptide.

for amino-acid analysis was hereafter performed in 6M HCl without mercaptoethanol.

Overall purification and yield

Treating these desacetyl-α-MSH-like peptides collectively as desacetyl-α-MSH, yield was 89% and the purification factor was about 14 000. (Yield was estimated by radioimmunoassay see section 3.11a). Thus desacetyl-α-MSH could be isolated in pure form with considerably less purification than MCH which was still impure after a 28 000 fold purification.

b) Purification of desacetyl-α-MSH-like peptides from lyophilised pituitaries

The purification of these peptides is summarised in the flow diagram (Fig 19).

The second peak of immunoreactive-α-MSH obtained from ion-exchange of a heated extract of the lyophilised pituitaries (Fig 8c) unlike that from the acetone-powder (Fig 8a) did not correspond to a peak of bio-activity. The fractions comprising this immunoreactive peak were pooled serarately from those of the following bioactive peak and each of these in turn was applied to Sephadex G-25; the resulting chromatograms were very similar (Fig 20).

Fig. 19. Flow diagram for the purification of desacetyl-α-MSH-like peptides from lyophilised pituitaries

N.B. mercaptoethanol was included during this purification at 0.1% v/v for extraction and 0.01% v/v for chromatography. The initial extract was prepared at 100°C.

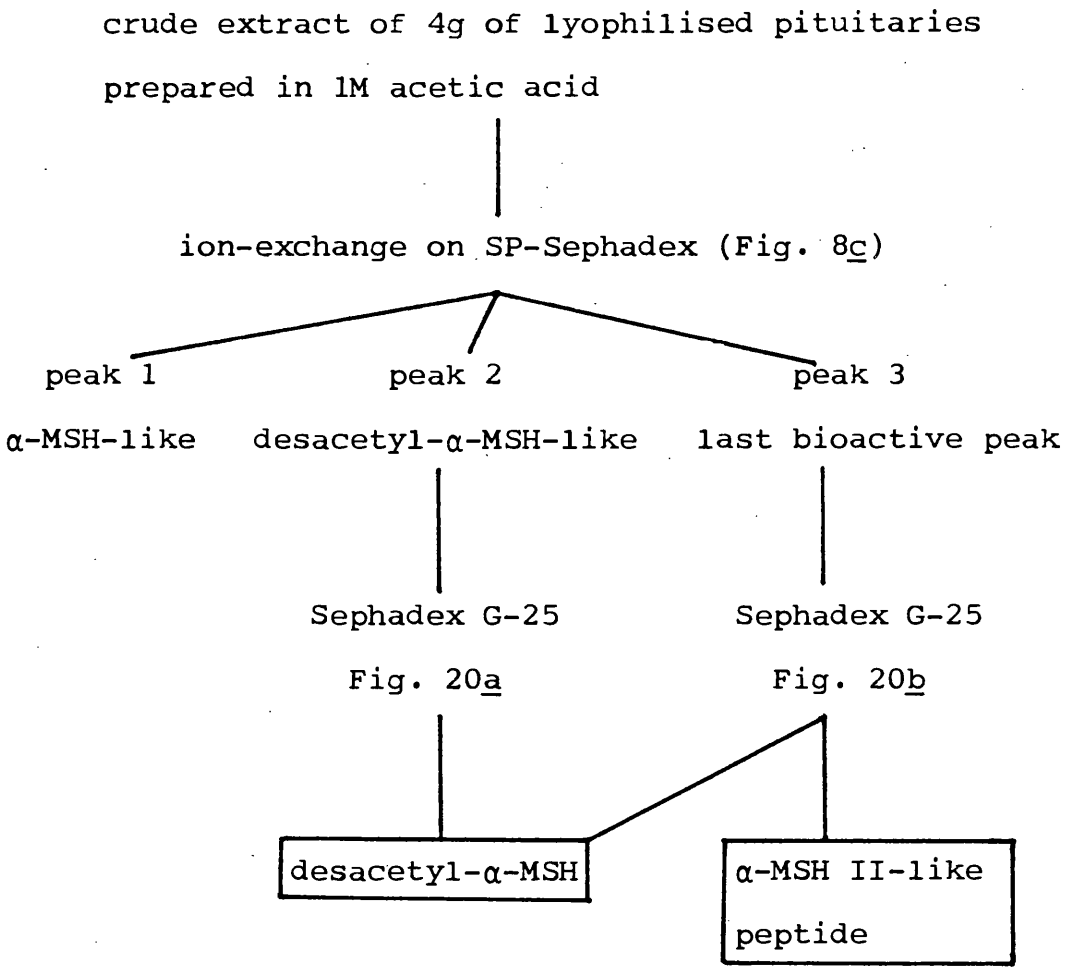
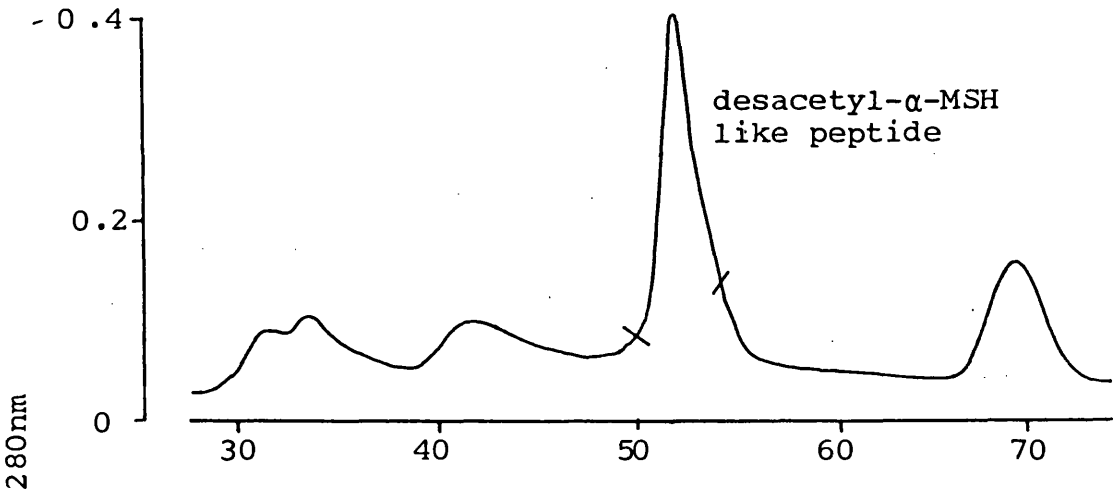


Fig 20

Gel-filtration of desacetyl-α-MSH-like peptides from ion-exchange of 4g of lyophilised pituitaries (Fig 8c) on Sephadex G-25. The second immunoreactive α-MSH peak (a) and the neighbouring bioactive MSH peak (b) from ion-exchange were lyophilised, re-constituted in 1.0ml of 1M acetic acid (including mercaptoethanol) and applied to a 77.5 X 1.1cm column of Sephadex G-25 eluted with the same solvent at 3.8ml h^{-1}. Fractions were pooled as indicated on the basis of the absorbance trace.

a immunoreactive α-MSH peak from ion-exchange



b bioactive MSH peak from ion-exchange

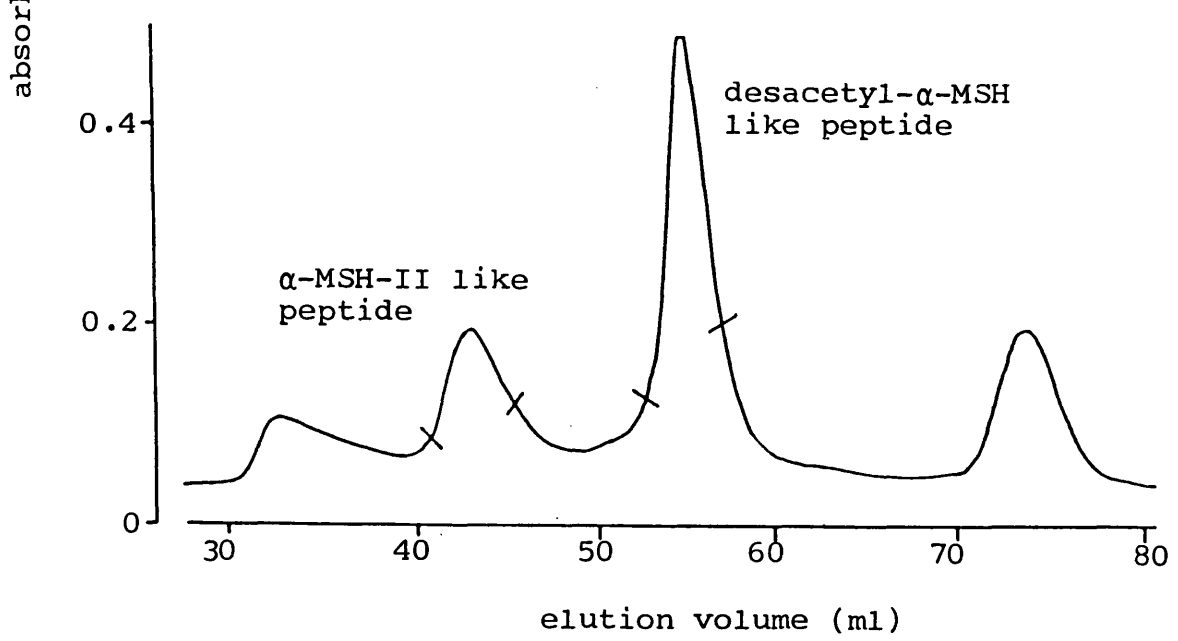


Table 7. Amino-acid composition and yields of desacetyl-α-MSH-like peptides purified from 4g of lyophilised pituitaries

	desacetyl-α-MSH-like peptides from Fig. 20		composition expected for α-MSH and desacetyl-α-MSH	α-MSH II like peptide from Fig. 20b	composition expected for α-MSH II
	Fig. 20a	Fig. 20b			
Ser	2.0	1.9	2	2.1	2
Tyr	0.9	0.8	1	0.9	1
Met	1.0	1.1	1	0.9	1
Glu	0.9	1.1	1	1.2	1
His	1.2	1.2	1	3.1	2
Phe	1.0	1.2	1	0.8	1
Arg	1.3	1.0	1	1.2	1
Trp	0.7	0.9	1	0.9	1
Gly	1.3	1.1	1	2.1	2
Lys	0.9	0.9	1	1.2	1
Pro	1.2	1.0	1	1.2	1
Val	0.9	0.9	1	N.D*	0
Ile	N.D*	N.D*		0.8	1
Amount recovered	637nmol (1.03mg)	892nmol (1.45mg)	-	463nmol (0.89mg)	-

Hydrolyses for amino-acid analyses were performed with 6M HCl and with 3M MESNA on samples of 3-5nmol of peptide. Analyses were performed on the autoanalyser (section 3.6)

*N.D = not detectable

Structural analysis

The peptide-peaks taken from Fig 20 were subjected to amino-acid analysis (Table7). Essentially integral ratios of residues were found indicating that these peptides were pure so they were not subjected to further purification by HPLC.

Hydrolysis in 6M HCl without mercaptoethanol showed no trace of methionine sulphoxide in any of these peptides suggesting that the presence of mercapto-ethanol during purification had either prevented or reversed methionine-oxidation.

The desacetyl-α-MSH like peptide common to both chromatograms of Fig 20 was desacetyl-α-MSH as shown by mass-spectrometry (see the next section).

No N-terminally shortened dervatives of desacetyl-α-MSH were found in this purification; since heat was used during the initial extraction, this supports the notion that those purified from the unheated extract of 2g of acetone-dried pituitaries are artifacts of proteolysis.

In addition to desacetyl-α-MSH, a higher molecular-weight (i.e. earlier eluting) peak was observed on gel-filtration (Fig 20b). This was not evident in the corresponding chromatogram from the acetone-powder

(Fig 17). Its amino-acid composition (Table 7) suggested that it was similar to α-MSH II - a pentadecapeptide purified from salmon pituitaries by Kawauchi et al (1980b):

α-MSH II

$CH_3C(=O)$—Ser-Tyr-Ser-Met-Glu-His-Phe-Arg-Trp-Gly-Lys-Pro-Ile-Gly-His-COOH

however, α-MSH II has only 2 His residues whereas this peptide has 3 (Table 7). Amino-acid analysis of solvent blanks showed traces of Gly, His, Lys and NH_3 where MESNA was used but only NH_3 where HCl was used. These contaminants were insufficient to account for the high His value which was determined in hydrolysates of both acids and it would appear that the α-MSH-II-like peptide does indeed have three His residues.

Overall purification and yield

From a heated extract of 4g of lyophilised pituitaries 2.48mg of pure undamaged desacetyl α-MSH was obtained which represents a yield of 103% and a purification factor of about 8 000. Thus purification of desacetyl α-MSH was easier from the lyophilised pituitaries than from the acetone powder (pf. 14 000) because of the preponderance of the desacetyl form of α-MSH and since heat precluded damage by proteolysis obviating the need

for further purification by HPLC.

Estimation of % yield or purification factor is not possible for the α-MSH-II-like peptide since there was no way of estimating it in the crude extract. (Desacetyl-α-MSH was estimated by radioimmunoassay after separation by ion-exchange, see section 3.11a). Similarly Kawauchi et al (1981) were unable to express their results in this fashion when they purified MSH's from salmon pituitaries. Therefore the results of this purification are compared to those of Kawauchi et al (1981) in Table 8 in terms of mg of peptide obtained per 100g wet weight of pituitaries:

Table 8

Yields of α-MSH's after purification from 4g of lyophilised pituitaries compared to those of Kawauchi et al (1981)

peptide	yield in mg/100g wet weight	
	this purification	Kawauchi et al (1981)
desacetyl-α-MSH	12	17
α-MSH-II	-	4
α-MSH-II-like peptide	4	-
α-MSH	(3)*	4

* calculated value assuming that the first immuno-reactive -α-MSH to elute from ion-exchange (Fig 8a) was α-MSH (see Fig 17b).

Thus the results of this purification were remarkably similar to those of Kawauchi et al (1981) though whether of not these are typical is uncertain since in the corresponding purification from 2g of acetone powder no α-MSH-II-like peptide was found and the acetylation status of α-MSH differed.

3.7 Fast-atom bombardment mass-spectrometry of des-acetyl-α-MSH like peptides purified from salmon pituitaries

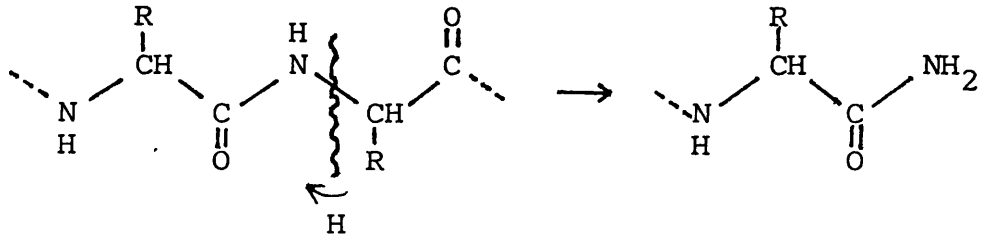
Introduction

The new technique of fast-atom bombardment (FAB) mass-spectrometry (Barber et al, 1981; Williams et al, 1981a,b) enables molecular-weight and sequence information to be obtained on nmol quantities of underivatised peptides. This method can determine directly the presence of labile substituent groups which might otherwise be changed or lost by conventional hydrolytic procedures or by the permethylation procedure used in electron-impact mass-spectrometry.

In FAB-spectra of peptides, the most abundant peak (excluding low-mass background) is usually that of the molecular ion. This enables accurate determination of molecular weights up to about 2 000 daltons. Of the types of fragmentation which occurr in FAB mass-spectrometry of peptides, the two most common and most useful have been identified (Williams et al, 1981b):

I

Charge retention by the N-terminal fragment



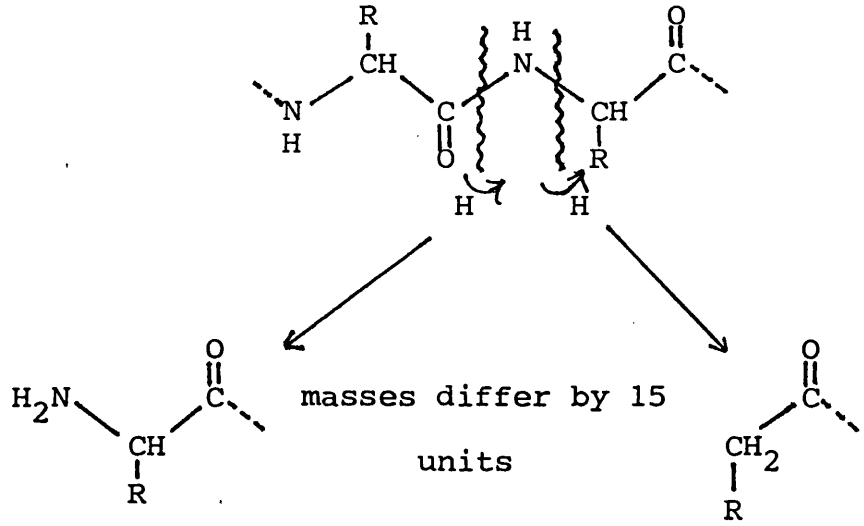
hydrogen migration

N-terminal amide sequence ion

NA

II

Charge retention by the C-terminal fragment



C-terminal amino sequence ion

CA

C-terminal alkyl sequence ion

C Alk

Whether the resulting sequence ions carry a net-negative or a net-positive charge is in part determined by the acid / base characteristics of the residues which they contain (Ions with net charges greater than unity are not seen in FAB spectra). Thus peptides containing a preponderance of basic residues (Arg, Lys and to a lesser extent His) generally give good positive ion spectra, conversely acidic residues favour negative ion spectra.

Whether N-terminal sequence-ions or C-terminal sequence-ions predominate depends on the distribution of charged sites in the peptide. In some cases the occurrence of both enables complete sequence determination.

a) Mass-spectrometry of peptides purified from lyophilised pituitaries

The FAB positive-ion mass-spectrum of the desacetyl-α-MSH like peptide from Fig 20a is shown (Fig 21). In this peptide, where basic residues predominate toward the C-terminal, only positively-charged C-terminal fragments (+ CA and + C Alk) are seen. The identification of such fragments is facilitated by their characteristic separation by 15 mass-units.

The molecular ion MH^+ at 1622 daltons is one mass-unit less than that predicted from the amino-acid composition

(Table 7). This mass-difference of 1 is perpetuated throughout the spectrum. It strongly indicates the existence of an amide group in the cryptic C-terminal portion of the peptide (i.e. the C-terminal 7 residues). From the amino-acid composition the Glx residue side-chain is the only site other than the C-terminal which could have borne an amide group; since this is defined by the mass-spectrum as Glu and not Gln - this peptide must be a C-terminal amide.

Since the molecular weight of this peptide differs by only one mass unit from that predicted by its amino-acid composition, we know that it does not contain an acetyl group at any site. e.g. α-MSH gives a molecular ion of 1664 daltons rather than 1622 daltons due to the presence of the acetyl group at the N-terminal.

The desacetyl-α-MSH like peptide from Fig 20b gave an identical spectrum to that shown in Fig 21.for the corresponding peptide of Fig 20a and since their amino-acid compositions were also identical it is concluded that they are the same . From the foregoing, the structure of this peptide has been determined as:

NH_2-Ser-Tyr-Ser-Met-Glu-His-

(Phe Arg Trp Gly Lys Pro Val)$CONH_2$

Brackets denote unspecified order.

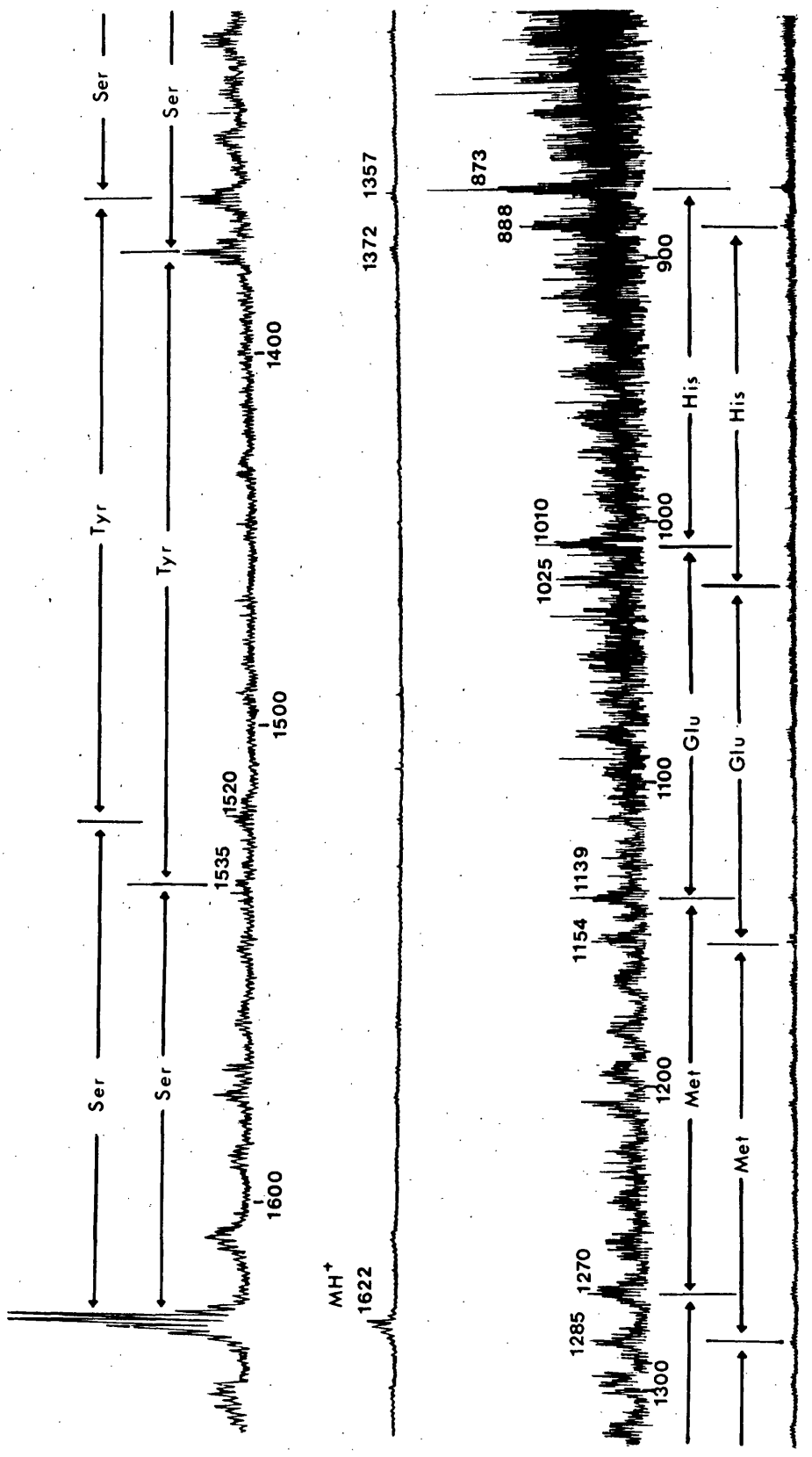
Fig 21

Fast-atom bombardment positive-ion mass-spectrum of the desacetyl-α-MSH-like peptide from Fig 20 a. This spectrum was obtained with a sample of about 10nmol. The major peak at m/z 1622 represents the intact peptide molecule carrying a single net positive charge, i.e. the molecular ion: MH^+. The characterisitic separation of sequence-ion peaks by 15 mass-units indicates that the main type of fragmentation occurring in this spectrum is of the second type described in the text, where charge is retained by the C-terminal fragment. These peaks therefore represent fragments of the molecular-ion which have lost various numbers of residues (0-6) from the N-terminal. The identity of these residues is deduced from the table below.

Table 9 Integral masses of amino-acid residues

Gly	57	Leu	113	Met	131
Ala	71	Ile	113	His	137
Ser	87	Asn	114	Phe	147
Pro	97	Asp	115	Met(O)	147
Val	99	Lys	128	Arg	156
Thr	101	Gln	128	Tyr	163
Cys	103	Glu	129	Trp	186

Met(O) = Methionine sulphoxide


Ser
Ser
Tyr
Ser
Tyr
1600
1535
1520
1500
1400
MH+
1622
1372
1357
873
888
900
1000
1010
1025
His
His
Glu
Glu
1100
1139
1154
Met
Met
1200
1270
1285
1300

It is very probable therefore that this is in fact desacetyl α-MSH:

NH_2-Ser-Tyr-Ser-Met-Glu-His-Phe-Arg-Trp-Gly-
Lys-Pro-Val-$CONH_2$

The α-MSH II like peptide from Fig 20b was similarly subjected to FAB mass-spectrometry, but no peptide attributable peaks were detected. According to its amino-acid composition the molecular weight of this peptide is 1881 daltons, not accounting for the possible presence of an acetyl group at the N-terminal which would increase this by 42 daltons. This is close to the present upper mass-limit of about 2 000 daltons beyond which ions are undetectable and this may explain failure to detect peptide-peaks in the mass-spectrum.

b) Mass-spectrometry of peptides purified from acetone-dried pituitaries

Positive-ion FAB mass-spectrometry of a 5nmol sample of peptide preparation A (see section 3.6a) gave a molecular ion (MH^+) of 1301 daltons and two sequence ions: + CA at 1154, and + C Alk at 1139 daltons due to the loss of a single residue from the N-terminal by type II fragmentation. The mass of the molecular ion was such that it could only have been caused by the major component of this peptide mixture, i.e. A (i), since the calculated mass of the other component

A(ii) was greater than 1301 daltons. However, the mass of the molecular ion was 15 daltons greater than that calculated for A(i) assuming it to be a C-terminal acid, or, 16 daltons greater assuming it to be a C-terminal amide (because COOH is 1 dalton greater than $CONH_2$). We know from the mass-difference between the molecular ion and the sequence ions that the mass of the N-terminal residue is 147 daltons. This, similarly, is 16 greater than that expected for an N-terminal methionine residue, and as such, could be due to either Phe or Met sulphoxide (see Table 9). It would appear therefore that the N-terminal residue was Met sulphoxide and since this accounts for a mass-difference of 16 and not 15, this indicates the presence of an amide rather than an acid group at the C-terminal.

3.8 A comparison of hypothalamic and pituitary forms of MCH and MSH

Rance and Baker (1979) have shown that MCH is present in the hypothalamus of Salmo gairdneri in equal or greater amounts than in the pituitary; the only indication that the two "pools" are connected in any way is their finding that the relative amounts vary according to background. Are hypothalamic and pituitary forms the same? - this could have important implications for the biological function of MCH (see discussion). Rance and Baker (personal communication) have already shown that both forms have the same mobility on polyacrylamide gel electrophoresis at acid pH. To study this further, hypothalamic and pituitary MCH's have been compared by gel-filtration and in each case the relationship to MSH's (bioactive and immunoreactive) has also been compared (Fig 22). Fresh tissue from trout (Salmo gairdneri) was used for these experiments because salmon hypothalami were not available.

Fig 22 shows that MCH from each source has the same apparent molecular weight, and that more was obtained from the hypothalamus. This is consistent with the hypothesis that the two pools are linked by axonal transport, but the relative abundance (more in the hypothalamus) is the converse of that expected for a neurohypophysial hormone (see section 1.5).

In the cases of both pituitary and hypothalmic extracts MCH co-elutes with the immunoreactive α-MSH peak - indicating that they are of similar molecular weight (non-identity has already been demonstrated by purification).

In the case of the pituitary extract, the α-MSH immunoreactivity eluted a little later than the peak of MSH bioactivity. This is probably because the bioactive peak is composed of a mixture of α- and β- MSH's, whereas the immunoreactive peak represents only the α-type MSH's (see section 3.3).

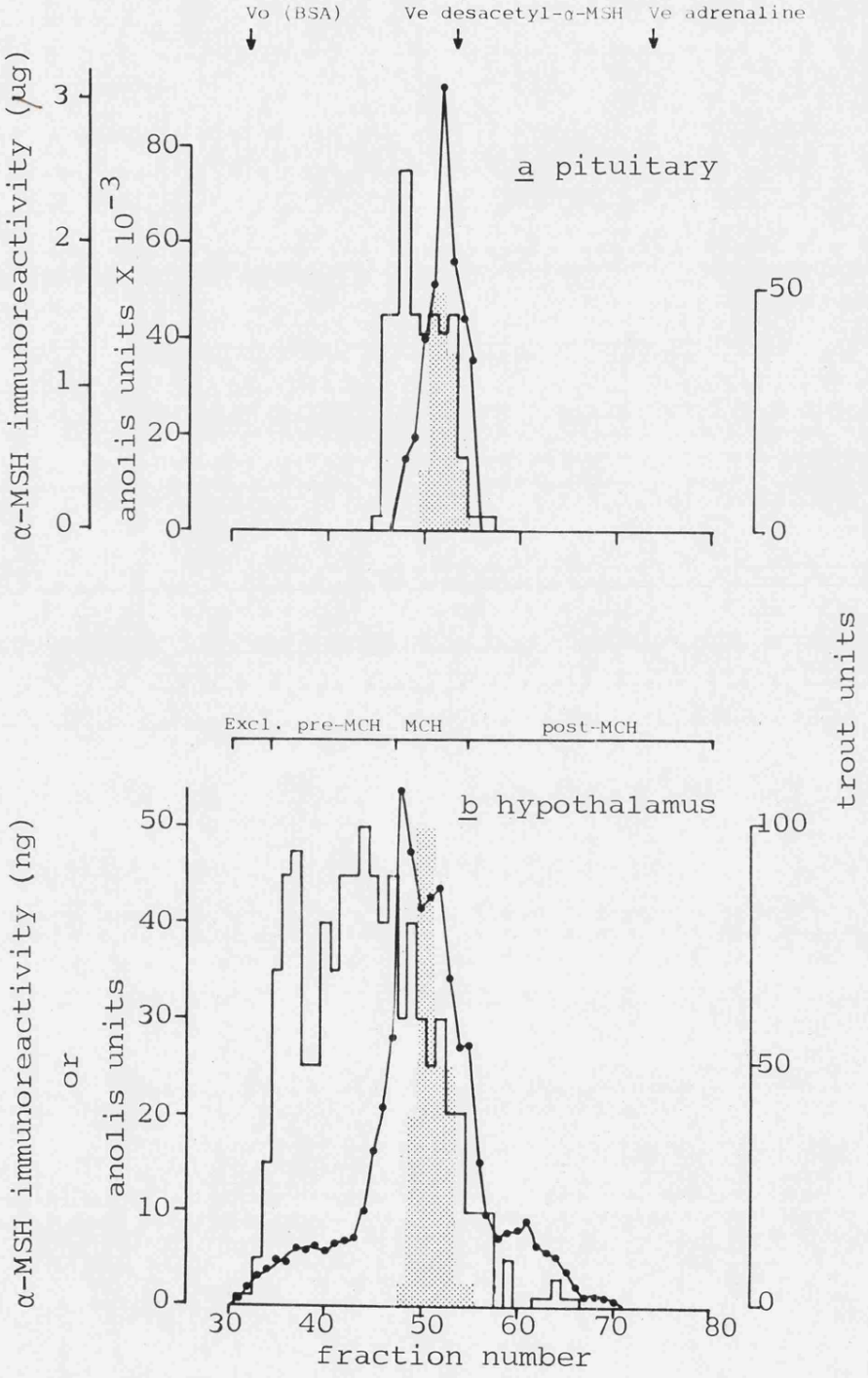
The hypothalamic immunoreactive α-MSH elutes in about the same position as desacetyl α-MSH , suggesting that some of the MSH bioactivity of the hypothalamus is attributable to α-MSH in its acetyl and / or desacetyl forms(Fig 22b).

The anatomical proximity of the hypothalamus and the pituitary and the very low amounts of MSH present in the hypothalamus relative to the pituitary make it eminently possible that hypothalamic "MSH" is in fact a contaminant from the pituitary. Although every precaution was taken to avoid this during their dissection and homogenisation - the possibility is still admissible. However, an observation which could not be explained by the occurrence of such contamination is the profound difference in molecular

weight distribution of bioactive MSH from the two sources. Although it could be argued that the hypothalamic immunoreactive α-MSH was due to contamination, this would not explain the higher molecular weight forms of bioactive MSH which occurr in the hypothalamus.

Fig 22

Comparison of pituitary (a) and hypothalamic (b) forms of MCH and MSH by gel-filtration on Sephadex G-25. Open histogram = MSH bioactivity; stippled histogram = MCH bioactivity; α-MSH immunoreactivity = •—• . The pituitaries and hypothalami of 35 rainbow trout were homogenised in boiling 1M acetic acid (10ml in each case). The pituitary extract was lyophilised, reconstituted in 1.0ml of 1M acetic acid and applied to the column (75 X 1.1cm) which was eluted at 4ml h^{-1} in the same solvent. Fraction volume was 1ml. The hypothalamic extract was treated identically except that, before lyophilisation, the homogenate was defatted with chloroform / methanol. The fish used in this experiment were 1-2 years old and were killed immediately after arrival at the laboratory. Mercaptoethanol was present at 0.1% v/v for extraction and 0.01% v/v for chromatography.


Vo (BSA)
Ve desacetyl-α-MSH
Ve adrenaline
α-MSH immunoreactivity (µg)
anolis units X 10⁻³
trout units
a pituitary
Excl. pre-MCH
MCH
post-MCH
b hypothalamus
α-MSH immunoreactivity (ng)
or
anolis units
fraction number

3.9 A hypothalamic inhibitor of MCH

Recovery of MCH from gel-filtration of the pituitary extract described in Fig 22a was about 80%. However, in an attempt to measure the recovery of MCH from the defatted hypothalamic extract of Fig 22b, no activity could be detected in a retained sample of the extract. This meant that the whole of the applied sample contained less than 7% of the activity recovered from gel-filtration. Occasional failure to detect MCH activity in crude hypothalamic extracts had previously been attributed simply to over-dilution of the extract, but it now appears that the activity may have been suppressed by an inhibitory factor.

To try to localise the "inhibitor" among the chromatographic fractions, four pools were made (Fig 22b): the exclusion peak; the MCH peak; and the fractions which eluted before and after MCH -"pre" and "post-MCH". Samples of 1/10 of the MCH peak were mixed with 1/10 of each pool. Another sample "complete re-mixture" was comprised of 1/10 of every pool. As control, 1/10 of the MCH pool was taken. Each of these samples was lyophilised (to remove acetic-acid and mercaptoethanol) and reconstituted in 50µl of MCH bioassay medium. The results of MCH bioassay of these samples are shown in Table 10.

Table 10

Localisation of MCH inhibitory factor among gel-filtration fractions

dilution of sample	control (MCH only)	MCH mixed with exclusion peak	MCH mixed with pre-MCH	MCH mixed with post-MCH	complete remixture
1	+	+	+	-	-
1/8	+	+	+	-	-
1/16	+/-	+/-	+	-	-
1/32	-	-	-	-	-

+ = strong response

+/- = threshold response

- = no response

Three scales were used for each determination

From Table 10 the inhibitor is clearly localised to to the post-MCH pool indicating it is of smaller molecular weight than MCH. The inhibitor was capable of reducing MCH activity by at least 16-fold. When all of the fractions were remixed, MCH activity was similarly abolished - confirming the original observation that activity in the crude extract was suppressed. Although the inhibitor can oppose the action of MCH on trout melanophores, whether it has intrinsic melanophore-dispersing activity was not determined. However, the inhibitor fraction was essentially free of Anolis melanophore-dispersing

activity (i.e. "MSH bioactivity"), suggesting that the inhibitor was not a type of MSH. The activity of MCH is not suppressed in crude pituitary extracts where α-, and β-MSH's are present in great excess of MCH; this also suggests that the inhibitor is not a type of MSH.

Having previously calibrated the gel-filtration column with adrenaline, we know that the inhibitor fraction would incorporate the catecholamine fraction of the hypothalamic extract. Although the usual response of teleost melanophores to catecholamines is one of melanin aggregation, Miyashita and Fujii (1975) have shown that very low concentrations of catecholamines can cause dispersion (see section 1.2a). Thus, the inhibitor could be a catecholamine(s) acting on β-adrenoceptors (since α-receptors were blocked by the presence of Rogitine in the bioassay medium). Time did not permit an investigation of the nature of the inhibitor, but this could be studied by testing its stability to proteases to see if it is a catecholamine or a peptide.

Although the physiological significance of this inhibitor is uncertain, it is important practically as it may explain why Kent (1959) was unable to detect MCH activity in the hypothalamus of Phoxinus

The realization of the existence of an inhibitor urges caution in deciding whether a tissue lacks MCH. Indeed a re-investigation of its phylogenetic occurrence may be warranted (e.g. can MCH be detected in mammalian hypothalamus after gel-filtration?).

More MCH can be detected in slices of hypothalamus (see Fig 1 in methods) than when fragments of hypothalamus - such as those used in this study are taken (Dr. B. Baker, personal communication). This might reflect an anatomical separation of nuclei containing MCH and the inhibitor.

3.10 Estimation of the molecular-weight of MCH

In section 3.8 crude extracts of trout pituitaries and hypothalami were chromatographed on Sephadex G-25 - a gel-filtration medium. How well a molecule penetrates into the gel beads is determined by its molecular size which for peptides (which do not have a stable three-dimensional conformation) is closely correlated with molecular weight. The degree of penetration is measured by determining the elution position of a peptide relative to that of a molecule which is totally excluded from the gel beads (in this case BSA). The resulting Ve/Vo values are inversely proportional to log molecular weight. This is how Fig 23 was constructed.

The data of Fig 22 were used to determine the elution position of MCH because conditions were optimal for the preservation of MCH - i.e. fresh material was used and extraction was performed in boiling acetic-acid. The column was operated with a peristaltic pump and elution positions were determined on the basis of time since this was found to be more reproducible than drop-count. The resulting molecular-weight estimate (1 700 daltons) is approximate only because too few standard peptides were available for calibration of the column.

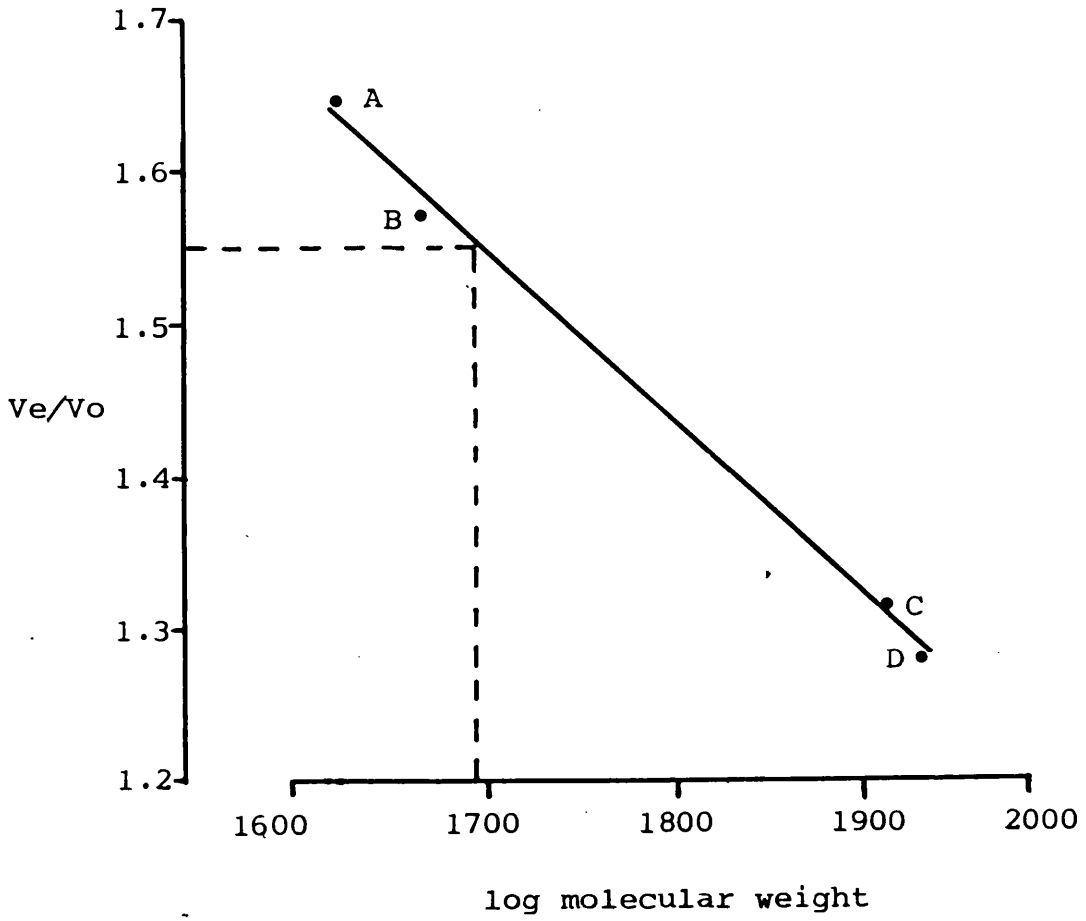


Fig. 23

Estimation of the molecular weight of MCH. The elution position of MCH on sephadex G-25 as determined in Fig. 22 was compared to that of various standard peptides of known molecular weight determined in separate runs on the same column. Ve, elution volume; Vo, void volume. A, desacetyl-α-MSH; B, α-MSH; C, α-MSH-II-like peptide; D, ACTH 1-16.

3.11 The use of radioimmunoassays for α-MSH and ACTH

Introduction

The pituitary content of any given hormone may be subject to large variation depending on the phsiological demand for it since it is a net function of rates of biosynthesis and secretion. It was deemed desirable therefore to compare the pituitary content of MCH to that of a variety of other peptide hormones. To this end, radioimmunoassays for α-MSH and ACTH were evaluated for use in measurement of the pituitary content of these peptides.

a) Immunoreactive α-MSH content of salmon pituitaries

Heated extracts of the commercial acetone-dried and lyophilised pituitary preperations (those described for Fig 7 b and c) were compared by radioimmunoassay for α-MSH. (Fig 24). Both extracts gave parallel competition curves suggesting similarity of the immunoreactive components of the extracts with the C-terminal end of α-MSH since the α-MSH antiserum is C terminally specific. Although this parallelism validates the quantitative use of the assay, this is so only in a relative sense, since 100% cross-reactivity is not a prerequisite of parallelism (see section 2.8 Table 1). In other words: although two values determined by radioimmunoassay may be compared to one another, in absolute terms (i.e. in μg) they

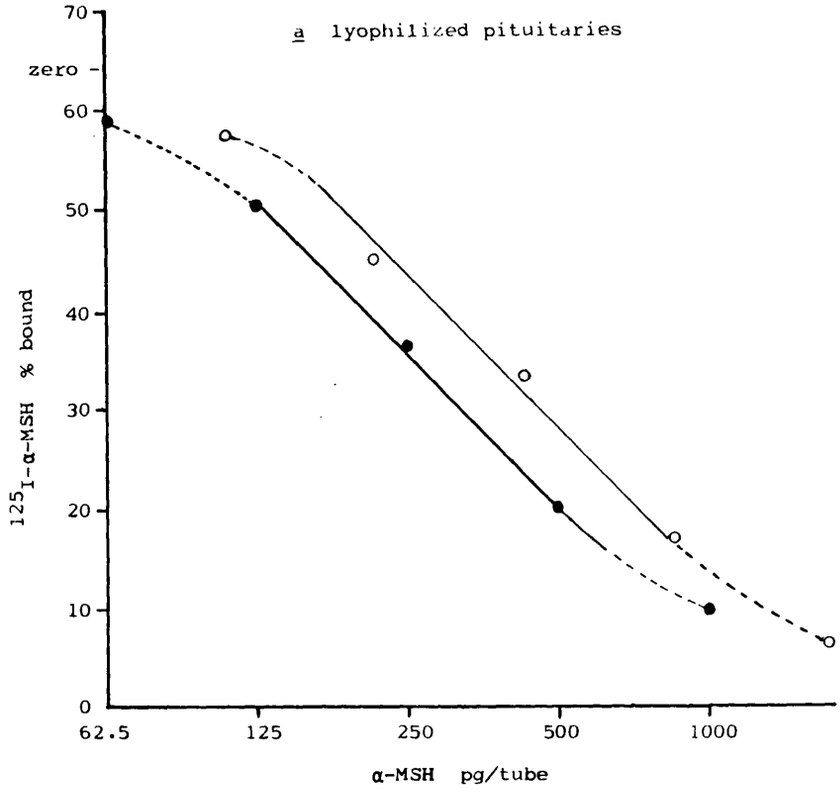
are liable to be underestimates.

Validation of the use of this assay in absolute terms comes from the realization that the major immunoreactive component of the extracts is desacetyl-α-MSH (see section 3.6) which has high (70%) crossreactivity with the antiserum. The other component is probably α-MSH (see section 3.6a)

Therefore in crude extracts this radioimmunoassay measures α-MSH and desacetyl-α-MSH together, and depending on their relative amounts, values are liable to be underestimated by up to 30% (because crossreactivity with desacetyl-α-MSH is 70%).

Estimates of the α-MSH content of the pituitary powders determined by assaying the extracts described for 7b and 7c and subject to the vagaries of the preceeding paragraph are given in Table 10 as "apparent α-MSH content". However, having separated α-MSH and desacetyl-α-MSH by ion-exchange (Fig 8) and knowing their crossreactivities (Table 2 : 100% and 70% respectively) it is possible to make a more accurate estimate of the amounts of each which exist in absolute terms (Table 11)

Fig. 24 Immunoreactive α-MSH of salmon pituitaries. Extracts of the commercial pituitary powders prepared in boiling acetic acid (i.e. those described for Fig. 7 b and c) were compared to synthetic α-MSH by radioimmunoassay. In each case, a 2-fold serial dilution of the extract (O) is compared to a similar series of α-MSH standards (●). Values are means of duplicate estimations. The zero-level on the y-axis denotes % ^{125}I-α-MSH bound in the absence of α-MSH. The extracts caused complete displacement of specifically-bound ^{125}I-α-MSH, and the slopes of the lines were parallel to the standard within the bounds of experimental error.


a lyophilized pituitaries
70
zero
60
50
40
30
20
10
0
125I-α-MSH % bound
62.5
125
250
500
1000
α-MSH pg/tube

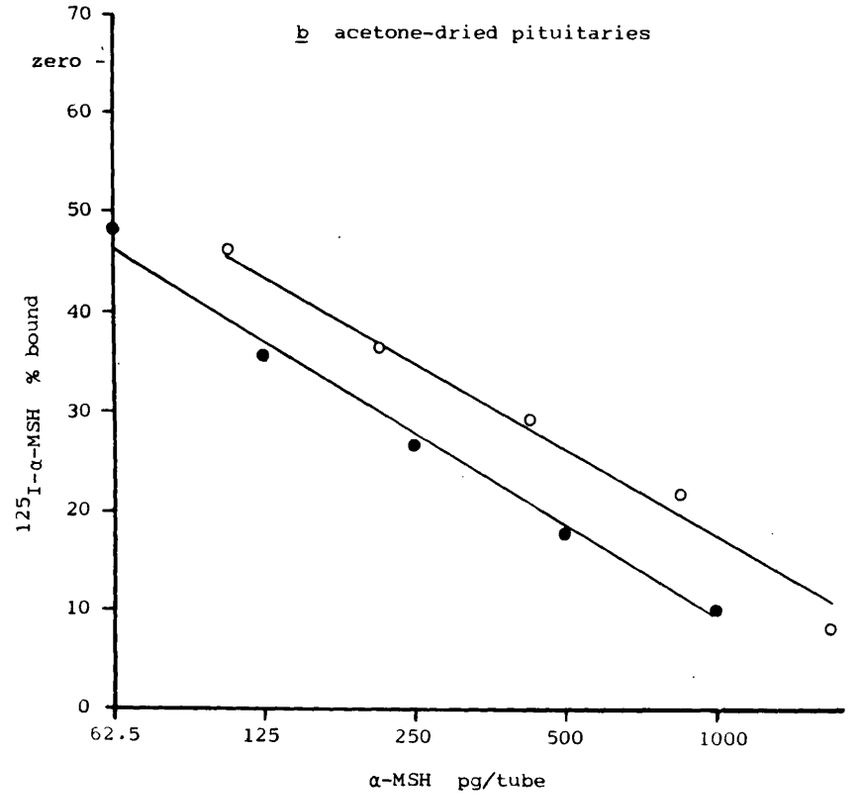

b acetone-dried pituitaries
70
zero
60
50
40
30
20
10
0
125I-α-MSH % bound
62.5
125
250
500
1000
α-MSH pg/tube

Table 11

Immunoreactive-α-MSH content of the commercial pituitary powders estimated by radioimmunoassay of heated extracts.

	peptide content $\mu g\ g^{-1}$		
	apparent-α-MSH*	α-MSH	desacetyl-α-MSH
acetone-dried pituitaries	448±32.6	197	358
lyophilised pituitaries	525±19.8	105	600

* Values are mean ± SEM, n=4 estimation on a single extract. 1g of pituitary powder = about 192 pituitaries = about 5g wet weight.

b) Immunoreactive ACTH content of salmon pituitaries

The unheated extract of acetone-dried piuitary powder described for Fig 8a was analysed for its content of immunoreactive ACTH using a commercial hACTH antiserum (Fig 25). The cross-reactivity of this antiserum with synthetic α-MSH at 50% displacement was found to be 6.5% in molar terms, and was probably due to the sequence-identity of α-MSH (except for its acetylated N-terminal) with the first 13 residues of hACTH. The specific binding of ^{125}I-pACTH to the antiserum could be completely prevented by α-MSH indicating that the antiserum was directed toward the N-terminal

region of hACTH.

Both α-MSH and the pituitary extract gave competition curves which differed in slope from the hACTH standard precluding measurement of the ACTH-content of the extract (Fig25). The slope of the piuitary-extract curve was closer to that of α-MSH than to that of the hACTH standard suggesting that the predominant immunoreactive component(s) of the extract were more akin to α-MSH than to hACTH. These results suggest that the ACTH-content of salmon pituitaries is low relative to that of α-MSH-like peptides, i.e. the ACTH immuno-reactivity detected here can be mostly accounted for by α-MSH and desacetyl-α-MSH.

These findings are in accord with those of Kawauchi et al (see 1982) who were unable to detect ACTH in salmon pituitary extracts (chemically rather than immunologically) despite having isolated two classes each of α-MSH and CLIP.

However, Scott and Baker (1975) using ACTH antisera which did not cross-react with α-MSH found comparable levels of α-MSH and ACTH in the pituitary of Salmo gairdneri by radioimmunoassay, and there is no doubt that teleost pituitaries contain ACTH.

Such differences could easily be explained by differences

in the physiological state of the animals when killed, e.g. factors such as stress and state of background adaptation might profoundly affect the relative amounts of these peptides.

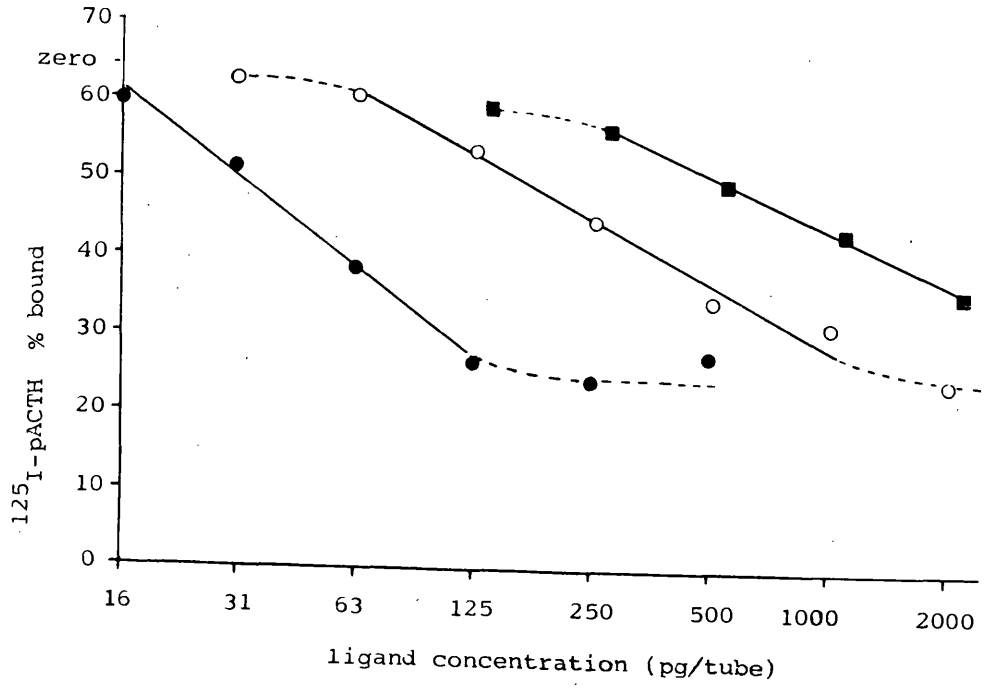


Fig 25

Immunoreactive "ACTH" of salmon pituitaries. An extract of the acetone-dried pituitary powder (i.e. that described for Fig 8a) was compared to hACTH and to α-MSH using the ACTH radioimmunoassay. A 2-fold serial dilution of the extract (■) is compared to similar series of hACTH (●), and the synthetic α-MSH (O) standards.

CHAPTER 4

DISCUSSION

4.1 The chemical nature of MCH

Since MCH was never isolated in pure form, direct knowledge of its chemical structure is not available. Nevertheless, certain deductions can be made about aspects of its chemical structure.

The concentrating activity of MCH on teleost melanophores, unlike that of catecholamines, appears to be insensitive to α-adrenergic blockade (section 3.5a). The activity of MCH is not decreased by oxidation with hydrogen peroxide (section 3.1d) - an observation which further contrasts it to the catecholamines which are readily inactivated by oxidation. Similarly, the inactivation of MCH by trypsin (section 3.1a) suggest it is a peptide and not a catechoalmine. This is also true of its apparent molecular-weight from gel-filtration (section 3.10) and its retention by hydrophobic HPLC media (e.g. section 3.2b). Thus the melanophore-concentrating activity of teleost pituitary extracts appears to be due to a peptide and not catecholamines.

Because the mobility of MCH in thin-layer electrophoresis is very similar to that of α-MSH (section 3.2d) and in view of the similarity in their molecular weights, it would appear that their net charges (at pH 4.4)

are similar. This would imply the existence of two or more of the basic residues (Arg, Lys or His) in the MCH molecule. This is consistent with the cationic behaviour of MCH during ion-exchange chromatography (section 3.3) and with its sensitivity to trypsin which suggests the presence of Arg and / or Lys (section 3.1a).

The gross physicochemical properties of MCH from cod pituitaries have recently been investigated by Bird-Westerfield et al (1980) using a qualitative in vivo bioassay based on the subjective assessment of pallor in the excised tails of Fundulus after intraperitoneal injection of pituitary extracts. They found MCH to be sensitive to trypsin, but not to reduction with borohydride - in common with the results of this study where MCH was found to be sensitive to TPCK-trypsin but not to reduction with thioglycollate (sections 3.1a and e respectively).

These workers were unable to detect MCH in extracts of whole brains of Fundulus. Since it would appear that they injected equal masses of pituitary and brain (in the form of extracts), it seems that they were comparing the concentration (rather than the amount) of MCH activity in cod pituitaries with that of whole brains of Fundulus. However, MCH is concentrated in the hypothalamus (section 2.2, Fig. 1) and consequently its concentration in whole

brain is relatively low. This may explain their failure to detect MCH in the brain. Alternatively the activity of MCH may have been masked by an inhibitory factor as was the case in section 3.9 where the MCH activity of a crude hypothalamic extract was undetectable.

4.2 Two forms of MCH in the pituitary

Ion exchange chromatography showed two peaks of MCH activity (Fig 8), so did HPLC (Fig 4); however, only one peak was ever observed on gel-filtration (Figs 3, 10 and 22). Since gel-filtration separates on the basis of molecular size, and ion-exchange and HPLC on the basis of charge and hydrophobicity respectively, it would appear therefore that two forms of MCH exist in the pituitary which are the same size but which differ in charge and hydrophobicity. We can but speculate as to what these differences are but acetylation and amidation are obvous possibilities: i.e. the existence of acetyl and desacetyl forms as in the the case of α-MSH and desacetyl-α-MSH, or of amide and acid forms.

4.3 Hypothalmic and pituitary forms of MCH

Many biologically active peptides are biosynthesised by proteolytic cleavage from higher molecular-weight precursor polypeptides and proteins. To compare hypothalamic and pituitary forms of MCH, gel-filtration

was used since this gives a clear indication of molecular size and would therefore show whether the MCH of the hypothalamus might be a precursor of pituitary MCH. The molecular-weight of MCH from each source was found to be the same (about 1700 daltons, section 3.10). Thus the MCH bioactivity of the hypothalamus was not due to a higher molecular weight precursor.

4.4 Is MCH a neurohypophysial hormone?

Two candidate neurohypophysial hormones are known to exist in teleosts : arginine vasotocin (AVT) and isotocin or icthyotocin (IT) (see Pickering and Heller 1969), however the biological roles of these hormones are less well-defined than those of the neurohypophysial hormones of mammals (Sawyer and Pang 1979). Thus MCH could be AVT or IT. Extracts of the pituitaries of Lampetra and Xenopus (which contain AVT) are devoid of MCH activity, although interestingly, hypothalamic extracts do have MCH activity (Rance and Baker, personal communication). It would appear therefore that MCH is not AVT. IT cannot be excluded in this way however, because both IT and MCH are absent from the pituitaries of all extra-teleostean species which have been tested.

All of the known neurohypophysial hormones are nonapeptides with a disulphide bridge between cysteine residues at positions 1 and 6. Furthermore, the

integrity of the disulphide bridge is essential to their biological activity. Reduction of this bridge with thioglycollate results in inactivation (see Vogt 1953; Follet and Heller 1964). In contrast, the activity of MCH is insensitive to reduction with thioglycollate (section 3.1e).

The molecular-weight of the neurohypophysial hormones (all less than about 1 100 daltons) is also inconsistent with MCH being a neurohypophysial hormone since its molecular weight (1700 daltons) is considerably greater than this. These results strongly suggest that MCH is not a neurohypophysial hormone.

Biologically active peptides other than the neurohypophysial hormones exist in the hypothalamo-neurohypophysial neurosecretory system of mammals - albeit in very much lower amounts than the neurohypophysial hormones: e.g. gastrin -17 (Rehfeld, 1978), CCK-8 (Beinfeld et al, 1980), Met - and Leu- enkephalin (Martin and Voigt, 1981), dynorphin (1-13) (Goldstein et al 1979). It has been suggested that such peptides may be involved in the control of the release of neurohypophysial hormones (e.g.Rossier et al, 1979).

Since MCH appears to be present in the hypothalmo-neurohypophysial neurosecretory system of teleosts and since it does not appear to be a neurohypophysial hormone, it may be related to these peptides.

4.5 The purification of MCH: contamination with MSH

In highly purified preparations of MCH there were still appreciable amounts of MSH bioactivity (Table 5). Thus it is possible that MCH - in addition to its capacity to cause concentration of teleost melanophores - also has intrinsic Anolis melanophore-dispersing activity. Alternatively, this could be due to residual contamination with an MSH-like peptide. This uncertainty will always be a problem inherent in using pituitaries as a source for the purification of MCH because of the great excess of Anolis melanophore-concentrating activity which exists in pituitary-extracts (in terms of dilution at which these activities can be detected in their respective bioassays).

It may be possible to avoid this by using hypothalamus rather than pituitary as a source for the purification of MCH. However, even in the hypothalamus there is more MSH bioactivity than MCH bioactivity (Fig 22b). However, this disparity is not nearly so great as it is in the pituitary (Fig 22a) and considering that the hypothalamus contains at least as much MCH as the pituitary, it probably represents a better source of MCH for the purpose of purification.

4.6 Do MCH and MSH act at the same receptor?

Pickford and Atz (1957), aware of the disparity between the amounts of teleost melanophore-concentrating activity and of frog melanophore-dispersing acitivity in extracts of teleost pituitary (seen in this case with *in vivo* bioassays) sought to explain this by supposing that MCH and MSH competed with one another, and that the observed response in each bioassay was a net effect of the two hormones. If this were true, then purification of MCH from MSH should result in an increase in the apparent acitivity of MCH. This was not the case (Table 5) which would suggest that MCH and MSH do not compete and may therefore act at different receptors. This would argue against similarity in the amino-acid sequence of MCH to the MSH's.

4.7 MCH as a neuropeptide

There is more MCH in the hypothalamus than in the pituitary; even in the hypothalamus (where MSH is considered to be a neuropeptide) there is more MSH bioactivity than MCH bioactivity; MCH is detectable in the hypothalamus but not in the pituitary of *Lampetra* and *Xenopus*. These observations (each already discussed in a different context) are more consistent with MCH being a neuropeptide involved in short range communication, rather than it being a hormone destined for the general circulation (although

these roles need not be mutually exclusive). The fact that MCH is active on melanophores in no way detracts from this possibility, on the contrary; melanophores bear more than a superficial resemblance to neurones and any substance which is active on melanophores is also potentially active on neurones. For example MSH: there is now a large amount of evidence which would suggest that MSH-like peptides of the brain (in addition to other peptides of the pro-opiocortin family) function as neuromodulators (see section 4.7).

It is interesting to speculate that MCH might be one of the known neuropeptides. Since MCH is present in both the hypothalamus and pituitary of teleosts, it may be involved in the control of the release of pituitary hormones. An obvious candidate role is the control of MSH secretion - since both MCH and MSH are found in the NIL and since the distribution of MCH between hypothalamus and pituitary varies according to background (Rance and Baker, 1979). However, present candidate peptides for the control of MSH secretion - which include fragments of oxytocin in mammals (see Jackson, 1979), and more recently TRH in the frog (Tonon et al, 1980), are too small to be MCH. Moreover, hypothalamic control of MSH secretion in teleosts so far appears to be aminergic (Goos and Terlou, 1977). The activity of MCH is not antagonised by Naloxone which would suggest it is not an opiate (Dr. B. Baker personal communication).

4.8 Hypothalamic MSH

Peptides of the pro-opiocortin family, including α-MSH, have been identified in the brains of mammals by radioimmunoassay, immunocytology, bioassay and by chromatographic characterisation. The highest concentrations of these peptides are found in the basal hypothalamus (as is the case for a number of other neuropeptides) and immunoreactive cell-bodies are confined to the region of the arcuate nucleus (for review see Krieger and Liotta , 1979).

Pro-opiocortin peptides have not yet been identified in the brains of teleosts, however, the discovery of immunoreactive-α-MSH and bioactive MSH in trout hypothalamus would suggest that they exist there (section 3.8).

Oliver and Porter (1978) studied the regional distribution of immunoreactive-α-MSH in the rat brain where they found it to be most concentrated in the pineal and hypothalamus. They have since shown by chromatographic characterization on HPLC that this immunoreactive-α-MSH is probably desacetyl-α-MSH (Porter et al, 1981). Gel-filtration on Sephadex G-25 of extracts from each region of the brain and from the NIL of the pituitary gave similar results: immunoreactive-α-MSH and Anolis bioactive MSH co-eluted in the same position as did synthetic α-MSH

chromatographed under the same conditions (Oliver and Porter, 1980).

Similar results were found in this study when whole trout pituitaries were chromatographed on Sephadex G-25 (Fig 22a). However, when trout hypothalamic fragments were treated in this way, a profound difference was observed in the bioactive MSH/immuno-reactive-α-MSH profiles; indeed, only a minor part of the Anolis bioactivity of the hypothalamus was attributable to immunoreactive-α-MSH - most of it apparently being due to higher molecular weight forms (Fig 22b). This might reflect a different manner of processing of the pro-opiocortin precursor in the teleostean hypothalamus compared to the PI of the NIL of the pituitary, but why this should be so is obscure.

The MSH's are biosynthesised from pro-opiocortin by specific proteolytic cleavage via a number of intermediates (see Lowry and Scott, 1977; Eberle 1980). Each of these, by definition, contains an MSH-sequence and as would be expected, these intermediates are generally of lower melanotropic potency than the MSH's per se (e.g. ACTH, which has very low potency relative to α-MSH in the Anolis assay - Tilders et al, 1975). Therefore the high molecular-weight bias of the molecular-weight distribution of trout hypothalamic "MSH" is probably underestimated by the Anolis bioassay.

A notable exception to this generalization is β-LPH which is about three-times as potent as α-MSH in the Anolis assay (Carter et al, 1979). Therefore trout β-LPH is an obvious candidate for one of the high-molecular-weight forms of hypothalamic "MSH".

4.9 Pituitary MSH

N-terminally shortened forms of desacetyl-α-MSH were isolated from unheated extracts but appeared to be absent from extracts which had been prepared at 100°C (section 3.6). This suggested that the shortened forms were artifacts of non-specific *in vitro* proteolysis which presumably were produced in the crude extract before proteases and MSH's were seperated by chromatography. Similar results were obtained by Lowry and Chadwick (1970) who found N-terminally shortened derivatives of dogfish (*Squalus acanthias*) α-MSH which they later concluded were artifacts (Bennet et al, 1974). Kawauchi et al (see 1981) have recently isolated N-terminally shortened forms of desacetyl-α-MSH from salmon (*Onchorynchus keta*) pituitaries but they found only small amounts of these peptides relative to desacetyl-α-MSH.

Although extraction in boiling acetic acid might produce artifacts, in this study it did not cause deamidation or de-acetylation, nor did it hydrolyse peptide-bonds of α-MSH. Since acetic acid can easily

be removed from the extract by lyophilisation, this might be a good general method for the extraction of basic peptides.

FAB mass-spectrometry proved very useful in the identification of purified MSH's and improvements in the instrumentation now in progress promise that this new technique will be widely used in the future.

4.10 Pro-opiocortin

Two types each of α-MSH (Kawauchi and Muramoto 1979; Kawauchi et al, 1980b), β-MSH (Kawauchi and Muramoto 1979; Kawauchi et al 1980a) and CLIP (Kawauchi et al 1982) have been isolated from salmon pituitaries. The sequences of these pairs of peptides show that they could not have been derived one from another by proteolytic cleavage, and it appears that two distinct precursor - i.e. pro-opiocortin - genes are expressed in salmon pituitary (see Kawauchi et al 1982).

Liotta et al(1980) have studied the products of pro-opiocortin in rat hypothalamus where it appears they resemble those of the PI more closely than those of the PD. However, it is unknown whether these differences represent differing post-translational processing of a common gene product, or the expression of different pro-opiocortin genes in hypothalamus and pituitary. The occurrence of a second pro-opiocortin gene in salmon pituitary is a

precedent for the latter hypothesis. Duplication of a common ancestral gene (perhaps originally expressed only in neurones) may have allowed the mutually independent evolution of two copies of the gene. It is even possible that the second pro-opiocortin gene of salmon is a hypothalamic form which - through mutation in gene expression - is now expressed in the pituitary also.

It would be interesting therefore to test the peptide products of the salmon pro-opiocortins (eg. α-MSH-II) for their behavioural effects when administered to the brain. Similarly, it would be interesting to study the distribution of these peptides in the brains of mammals and teleosts by immunostaining. Study of the hormonal roles of the respective gene-products in salmonids by radioimmunoassay of pituitary and plasma should also prove interesting.

CONCLUSION

Attempts to purify MCH from teleost pituitaries were unsuccessful. Using the same methods, pure undamaged α-MSH's were obtained in high yield. Contamination of highly purified MCH with MSH bioactivity suggested that the hypothalamus rather than the pituitary would be a better source for purification.

The possibility that MCH is a neuromodulator rather than a neurohormone indicates that kilogram rather than gram quantities of fresh tissue might be necessary to purify it. The realization that its activity can be masked in crude hypothalamic extracts urges caution in deciding whether a tissue lacks MCH and this may warrant a re-investigation of its occurrence in extra-teleostean species. Many neuropeptides are found outside the central nervous system (e.g. TRH is found in high concentration in amphibian skin (Yasuhara and Nakajima, 1975)) and similarly this would urge a re-investigation of its tissue-occurrence.

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